EVALUATION OF ANTIFUNGAL EFFICACY OF SODIUM HYPOCHLORITE (NaOCl, 5.25%), ETHYLENE DIAMINE TETRA ACETIC ACID (EDTA, 17%) AND SALINE WITH FOUR DIFFERENT ANTIFUNGAL AGENTS ON *CANDIDA ALBICANS*: AN *IN VITRO* STUDY

Dissertation submitted to

The TamilNadu Dr M.G.R. Medical University

In Partial fulfillment of the degree of

MASTER OF DENTAL SURGERY

BRANCH IV

CONSERVATIVE DENTISTRY

AND ENDODONTICS

2013-2016

CERTIFICATE

This is to certify that this dissertation titled ***"Evaluation of Antifungal efficacy of Sodium Hypochlorite (NaOCl, 5.25%), Ethylene Diamine Tetra Acetic acid (EDTA, 17%) and Saline with Four Different Antifungal Agents on Candida albicans: An In vitro study"*** *is a bonafide record of the work done by Dr.Betty Babu under our guidance during her post graduate study during the period of 2013-2016 under* **THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY, CHENNAI**, *in partial fulfillment for the degree of* **MASTER OF DENTAL SURGERY IN CONSERVATIVE DENTISTRY & ENDODONTICS, BRANCH IV**. *It has not been submitted (partial or full) for the award of any other degree or diploma.*

Dr. S. Rajesh MDS.
Professor & Head
Dept. of Conservative Dentistry and Endodontics
Sree Mookambika Institute of Dental Sciences
Kulashekaram

Dr. Mano Christaine Angelo MDS,
Professor
Dept of Conservative Dentistry & Endodontics
Sree Mookambika Institute of Dental Sciences
Kulasekharam - 629 161

Dr. RAJESH S; M.D.S
Professor, HOD& Guide
Dept. of Conservative
Dentistry & Endodontics

Dr.Mano Christaine Angelo; M.D.S
Professor & Co-guide
Dept. of Conservative
Dentistry & Endodontics

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

KULASEKHARAM

ENDORSEMENT BY THE PRINCIPAL/ HEAD OF THE INSTITUTION

This is to certify that the dissertation titled **"EVALUATION OF ANTIFUNGAL EFFICACY OF SODIUM HYPOCHLORITE (NaOCl, 5.25%), ETHYLENE DIAMINE TETRA ACETIC ACID (EDTA, 17%) AND SALINE WITH FOUR DIFFERENT ANTIFUNGAL AGENTS ON *CANDIDA ALBICANS*: AN *IN VITRO* STUDY"** is a bonafide research work done by **Dr. BETTY BABU** under the guidance of **Dr.RAJESH S, M.D.S**, Professor &HOD, Department of Conservative Dentistry and Endodontics, Sree Mookambika Institute of Dental Sciences, Kulasekharam.

17.12.15

Dr. Elizabeth Koshi M.D.S.
Principal
Sree Mookambika Institute of Dental Sciences
Kulashekaram, K.K. Dist.

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

Date: 17.12.15

DR.ELIZABETH KOSHI; M.D.S
Principal
Sree Mookambika Institute of Dental Sciences
VPM Hospital Complex, Padanilam
Kanyakumari District,
Tamilnadu- 629161

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

KULASEKHARAM, KANYAKUMARI DIST., TAMIL NADU, INDIA.

INSTITUTIONAL RESEARCH COMMITTEE

Certificate

This is to certify that the research project protocol, ***Ref no. 05/08/2014***titled, ***"Evaluation of antifungal efficacy of Sodium Hypochlorite (NaOCl,5.25%), Ethylene Diamine Tetra Acetic Acid (EDTA, 17%) and Saline with four different antifungal agents on Candida albicans: an in vitro study"*** submitted by ***Dr.Betty Babu, IIYear MDS, Department of Conservative Dentistry and Endodontics***has been approved by the Institutional Research Committee at its meeting held on 12th ***August 2014.***

Convener
Dr. T. Sreelal

Secretary
Dr. Anuroopa A.

Sree Mookambika Institute of Medical Sciences
Kulasekharam (K.K District, TN) 629161

Phone No: 04651-280866, Fax No. 04651-280740

Institutional Human Ethics Committee

Registered under CDSCO with Reg No. ECR/416/Inst/TN/2013

Ref. No. SMIMS/IHEC/2015/A/04 **Date: 10th April 2015**

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This is to certify that the Research Protocol Ref. No. **SMIMS/IHEC/2015/A/04**, entitled "Evaluation of Antifungal Efficacy of NaOCl [5.25%], EDTA [17%] and Saline With Combination of Different Antifungal Agents on *Candida albicans*: An *In Vitro* Study" submitted by Dr. Betty Babu, Postgraduate of Department of Conservative Dentistry and Endodontics, SMIDS has been approved by the Institutional Human Ethics Committee at its meeting held on 13th of March 2015.

[This Institutional Human Ethics Committee is organized and operates according to the requirements of ICH-GCP/GLP guidelines and requirements of the Amended Schedule-Y of Drugs and Cosmetics Act, 1940 and Rules 1945 of Government of India.]

IHEC Date 10/4/15

Dr. Rema Menon. N
Member Secretary
Institutional Human Ethics Committee
Professor of Pharmacology and HOD
SMIMS, Kulasekharam [K.K District]
Tamil Nadu -629161

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"I can do all this through him who gives me strength"

CONTENTS

LIST OF ABBREVIATIONS

NaOCl- Sodium hypochlorite	NiTi- Nickel Titanium
EDTA- Ethylene diamine tetra acetic acid	i.e.- that is
GIC- Glass Ionomer Cement	Fig.- Figure
DNA- Deoxyribo nucleic acid	SEM- Scanning electron microscopy
CFU- Colony Forming Units	H_2O_2- Hydrogen peroxide
ATCC- American Type Culture Collection	AMB- Amphotericin B
RVG- Radiovisiography	Cs-np – Chitosan nanoparticles
RNA- Riboxy nucleic acid	ZnO-np- Zinc oxide nanoparticles
$AgNO_3$-Silver nitrate	°C – Degree celcius
Cts- Chitosan	PBS- Phosphate buffered saline
AgNP – Silver nanoparticles	MIC- Minimum inhibitory concentration
Ag- Silver	PCR- Polymerase chain reaction
µm-Micrometer	HIV- Human immune deficiency virus
ANOVA- Analysis of Variance	AIDS- Acquired Immune deficiency syndrome
SPSS-Statistical package for social sciences	ppm- Parts per million
HOCl- Hypochlorous acid	$Ca(OH)_2$- Calcium hydroxide
OCl^-- Hypochorite ions	PVA- Poly vinyl alcohol
C.albicans- Candida albicans	µg/ml – Microgram per millilitre
NYT- Nystatin	MRSA- Methicillin resistant *staphylococcus aureus*

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Endodontic infections are polymicrobial in nature with predominance towards anaerobic species. Microbes are associated virtually with all diseases of the pulp and periradicular tissues. The microbial ecosystem of infected root canals may consist of bacteria, including spirochetes and fungi. Polymicrobial interactions and nutritional requirements make the cultivation and identification of all organisms from endodontic infections very difficult.[1,2]

Among the microorganisms, fungi play an important role in the failed endodontic treatments.[1,2]Fungi are common opportunistic pathogens in the oral cavity. Approximately one- third of individuals with no oral disease carry fungi. Fungi are eukaryotic microorganisms that exhibit two basic structural forms: a "yeast form" (unicellular) and a "mould form" (multicellular).The most important oral fungi belong to genus *Candida* with *Candida albicans* being the most predominant and commonly isolated yeast from the oral cavity followed by *Candida glabarta, Candida krusei, Candida tropicalis* and *Candida kefyr*.[3] The incidence of *Candida albicans* in oral cavity has been reported to be 30% to 45% in healthy adults and 95% in patients infected with human immunodeficiency virus. The transition of *Candida albicans* from a harmless commensal to a pathogenic organism appears to be dependent on minor changes in predisposing conditions that cause the expression of a variety of virulence.[4]

Candida albicans is a dimorphic fungus that exists in many morphologic forms such as germ tubes, blastospores, pseudohyphae, true hyphae and chlamydospores.[5,6]Each form of growth depend on environmental conditions such as the pH level, temperature and nutritional source.[3]*Candida* has the ability to grow on

the dentinal surfaces in the absence of oral tissue fluids and penetrate into the dentinal tubules by its various growth patterns. Sen *et al*. suggested that candida be considered as "dentinophilic" microorganism.[4]

The goal of endodontic treatment is to achieve the disinfection of root canals by the eradication of microbes and prevention of reinfection by proper cleaning and shaping along with the use of irrigants to remove the inflamed and necrotic tissue, microbes/ biofilms and other debris from the root canal space.[1]

Irrigation during chemomechanical preparation in endodontic treatment is just as essential as any other step.[7] Irrigants prevent packing of the hard and soft tissue in the apical root canal. Irrigants are used in endodontic procedures not just as an antimicrobial agent but also to lubricate the dentinal walls. The effectiveness of irrigation depends on the working mechanisms of the irrigant and the ability to bring the irrigant in contact with the microorganisms and tissue debris inside the root canal.[8]

Sodium hypochlorite (NaOCl) was introduced as part of endodontic treatment in 1936 by Walker. NaOCl is one of the most popular and widely used endodontic irrigants due to its antibacterial activity and its ability to dissolve necrotic tissue remnants. NaOCl is both an oxidizing and hydrolyzing agent. It is commonly used in concentrations between 0.5% and 6%. [9,10]Sodium hypochlorite at concentration 5.25% has been proved to be most effective in removing endodontic biofilm.[11]

Ethylene Diamine Tetraacetic acid (17% EDTA) is a chelating agent introduced by Nygaard – Østby in 1957. It effectively removes smear layer by chelating the inorganic component of the dentin.[7]

Baker *et al*. advocated the use of physiological saline. Saline accomplishes gross debridement and lubrication. Irrigation with saline sacrifices chemical destruction of microbiologic matter and dissolution of mechanically inaccessible tissues.[7]

Chitosan, a natural polysaccharide, is a derivative of chitin, which is commonly found in shells and exoskeletons of some crustacean and is the second most abundant bio-polymer with unique structural and physiologic characteristics.[12]Chitosan exhibits a broad spectrum of antimicrobial activity by binding to the negatively charged bacterial cellwall followed by attachment to the DNA, inhibiting its replication. Chitosan is a biopolymer having antibacterial properties.[13]

Silver nanoparticles have high therapeutic potential. Silver nanoparticles have a wide range of antimicrobial activities and exhibit high performance even at a very low concentration.[14]Chitosan- silver nanocomposite is seen to possess a capability of being used as a biosensor as well as in the treatment of cancer as the chitosan present in the nanocomposite is very specific to the cancer cells. Chitosan stabilizes and prolongs the action of silver.[15]

Clotrimazole, an imidazole derivative, is primarily used locally in the treatment of vaginal and skin infections due to yeasts and dermatophytes. Clotrimazole works to kill individual *candida* or fungal cells by altering the permeability of fungal cell wall. Clotrimazole has been used successfully in patients who had failed to respond to other antifungal agents such as Nystatin and Amphotericin B.

Fluconazole is a synthetic, bistriazole antifungal agent, effective in treating superficial and systemic infections caused by *candida* species. Fluconazole works by interfering with synthesis of fungal cell membrane. Fluconazole is commonly used to prevent yeast infections in patients undergoing bone marrow transplantation.

Amphotericin B is a polyene antifungal agent with activity against wide variety of fungal pathogens. Amphotericin B exerts its antifungal effect by disruption of fungal cell wall synthesis. As with other polyene antifungals, Amphotericin B binds with ergosterol, a component of fungal cell membranes, forming a transmembrane channel that lead to monovalent ion (K^+, Na^+, H^+ and Cl^-) leakage, which is primary effect leading to fungal cell death.

The success of root canal treatment largely depends on the elimination of microbial contamination from the root canal system, although mechanical instrumentation can reduce bacterial population, effective elimination of bacteria cannot be achieved without the use of antimicrobial root canal irrigation and medication.[16]The number of colony-forming units (CFUs) in an infected root canal is usually between 10^2 and 10^8. A positive correlation exists between an increase in size of the periapical radiolucency and both the number of bacteria species and CFUs present in the root canal. The use of improved culturing and molecular methods now detect the presence of many more organisms in endodontic infections than previously determined.[3]

In this present study four antifungal agents have been used to assess the antifungal efficacy against *Candida albicans*. The study hypothesis was that chitosan-silver nanocomposite has better antifungal efficacy against *Candida albicans.*

AIMS & OBJECTIVES

AIM:-

The aim of this *in vitro* study was to evaluate the efficacy of NaOCl 5.25%, EDTA 17% and saline as final irrigant with the inclusion of antifungal agents [Chitosan-silver nanocomposite (20%Ag), Fluconazole (0.2%), Clotrimazole (1%) and Amphotericin B (0.2%)] on *Candida albicans.*

OBJECTIVES:-

- Evaluation of antifungal activity of Chitosan silver nanocomposite.
- Comparison of antifungal efficacy of Nano particles with standard antifungal agents.
- To evaluate which among the root canal irrigant gives better efficacy in the elimination of *Candida albicans*.

REVIEW OF LITERATURE

Smith *et al.* 1971[17] in an *in vitro* study evaluated the antifungal activity of clotrimazole (Bay b 5097) compared with those of Amphotericin B, Griseofulvin, nystatin, and pyrrolnitrin. They reported that clotrimazole is less active than amphotericin B against *Candida albicans* and *Aspergillus fumigatus*. The activity of clotrimazole against dermatophytes is comparable to that of pyrrolnitrin and superior to that of either nystatin or griseofulvin. More importantly, the ranges of inhibitory and fungicidal concentrations are narrow with little deviation either within or between species.

Nair *et al*. 1990[18] in a follow-up study, used light and electron microscopy to analyse nine therapy-resistant and asymptomatic human periapical lesions, which were removed as block biopsies during surgical treatment of the affected teeth. They reported that in majority of root-filled human teeth with therapy-resistant periapical lesions, microorganisms may persist and may play a significant role in endodontic treatment failures.

Kawasaki *et al*. 1991[19] examined *in vivo* efficacy and *in vitro* activity of fluconazole, a novel triazole antifungal agent, and obtained results which are summarized as follows: 1. Fluconazole showed a higher serum concentration than ketoconazole after oral administration to mice. The 50% effective dose of fluconazole administered orally to mice was similar to that of fluconazole injected to mice intraperitoneally in a systemic candidiasis model. 2. Prophylactic effects of fluconazole were excellent against systemic candidiasis, cryptococcosis and aspergillosis in mice in comparison with those of ketoconazole and miconazole. 3. The multiple administration of fluconazole effectively decreased the number of viable cells of *Candida albicans* colonized in kidneys of mice when the serum level of

fluconazole was kept to exceed its IC99 values against the inoculated pathogen. Thus, a good correlation between the *in vitro* activity of fluconazole and its *in vivo* efficacy was confirmed. *In vivo* efficacies of ketoconazole and miconazole, however, failed to reflect their marked *in vitro* activities. 4. *C. albicans* No. 32 developed no drug-resistance to fluconazole during transfers in medium containing fluconazole at a concentration of 1 micrograms/ml.

Gilbert *et al*. 1997[20] in a review article focuses on biofilm susceptibility to antimicrobials and its mechanisms that are associated with resistance. The dominant mechanisms they highlighted are:- (i) modified nutrient environments and suppression of growth rate within the biofilm; (ii) direct interactions between the exopolymer matrices, and their constituents, and antimicrobials, affecting diffusion and availability; and (iii) the development of biofilm/attachment-specific phenotypes.

Sen *et al*. 1997[4] investigated the growth patterns of *Candida albicans* in relation to the human radicular dentin and root sections were infected with *C. albicans* grown in calf serum and incubated for various periods. The sections were fixed in glutaraldehyde, split into two halves, and evaluated by scanning electron microscopy and then observed blastospores and hyphal structures on the root canal wall of all specimens. Most of these structures, particularly pseudohyphae, exhibited penetration into dentinal tubules.

Clarkson *et al.* 1998[21] has reviewed the chemical properties and production of commercial sodium hypochlorite. Production of domestic bleaches and infant sanitizer was compared in terms of cost and ease of use. Brief guidelines for clinical use, storage, handling and disposal are as follows: always use freshly prepared solutions, use only demineralized water for dilution, store solutions in opaque glass or coated polyethylene containers which are tightly sealed, use Luer- Lok plastic

syringes to prevent dislodgement of the needle, other accidents. Do not inject forcibly, or allow needle to bind in canal, always use rubber dam during endodontic treatment and ensure that it maintains a tight seal against the tooth and gingiva. Discard syringes and unused solutions at the end of the appointment, flush drains with copious quantities of water.

Michael *et al*. 1999[22] reviewed the use of Fluconazole and Itraconazole in the treatment of *Candida albicans* infections. Fluconazole exhibits predictable pharmacokinetics and is effective, well tolerated and suitable for use in most patients with *C. albicans* infections. Prophylactic administration of Fluconazole can help to prevent fungal infections in patients receiving cytotoxic cancer therapy. The increasing use of Fluconazole for the long-term prophylaxis and treatment of recurrent oral candidosis in AIDS patients has led to the emergence of *C. albicans* infections that are not responsive to conventional doses. Wider spectrum antifungal, such as Itraconazole, should be sought if treatment with fluconazole fails. Itraconazole solution is as effective as Fluconazole. Itraconazole solution can be effective in AIDS patients with *C. albicans* infections that are non-responsive to Fluconazole.

Sen *et al*. 1999[23] tested the antifungal properties of 0.12% CHX, 0.5% NaOCl and 5% NaOCl against *C.albicans*. The root sections were enlarged and smear layer was removed in half of the specimens. The root canal was dispensed with inoculums of *C.albicans*. After 10 days, the root sections were treated with 3ml of either disinfectant. Then root sections were incubated at 37˚C for 24 hours and then they concluded that *C.albicans* was more resistant to these irrigating solutions when smear layer was present. When the smear layer was absent, NaOCl displayed antifungal activity for 30 minutes.

Waltimo *et al.* 1999[24] evaluate the susceptibility of seven strains of *Candida albicans* to four disinfectants: iodine potassium iodide, chlorhexidine acetate, sodium hypochlorite and calcium hydroxide. In addition, all possible pairs of the disinfectants were tested in order to compare the effect of the combination and its components. The results showed that sodium hypochlorite, iodine potassium iodide and chlorhexidine acetate are more effective than calcium hydroxide against *C. albicans*.

Ayhan *et al.* 1999[25] evaluated the antimicrobial effect of various endodontic irrigants against six selected microorganisms. *Staphylococcus aureus, Enterococcus faecalis, Streptococcus salivarius, Str.pyogenes, Escherichia coli and Candida albicans*. The result showed that 5.25% NaOCl was superior in its antimicrobial abilities compared with other irrigants used. A reduced concentration of NaOCl (0.5%) resulted in significantly decreased antimicrobial effects. When compared with 21% alcohol, 0.5% NaOCl and 2% chlorhexidine, paramonochlorophenol (cresophene) showed a greater antimicrobial effect.

Sen *et al.* 2000[26]evaluated the antifungal effect of ethylene diamine tetraacetic acid (EDTA) on *Candida albicans*, comparing it with that of various disinfectants and common antifungal agents. Two clinical oral isolates and 1 standard strain of *C. albicans* were included in this study. Main contents of the test solutions were sodium hypochlorite, EDTA, chlorhexidine, hexetidine, benzalkonium chloride, povidone-iodine, nystatin, and ketoconazole. The agar diffusion method was used to determine the antifungal effects of the solutions. The specimens of EDTA demonstrated the highest antifungal activity in comparison with routine antifungal drugs and all other solutions.

Baumgartner *et al.* 2000[27] evaluated the presence of *Candida albicans* in the contents of infected root canals and aspirates of cellulitis/abscesses of endodontic origin using the PCR. They reported the presence of *C. albicans* in 5 of 24 (21%) samples taken from root canals, but none was detected in the periradicular aspirates and this indicates that PCR is an extremely sensitive molecular method that may be used to identify *C. albicans* directly in samples from infections of endodontic origin.

Rene *et al.* 2000[28] investigated the potential emergence of resistance to Clotrimazole in a prospectively monitored HIV-infected paediatric population receiving this azole. They concluded that resistance to Clotrimazole develops in isolates of *C. albicans* from HIV infected children and cross- resistance to other azoles may develop concomitantly with this resistance correlating to refractory mucosal candidiasis.

Spratt *et al.* 2001[29] evaluated the bactericidal effect of four antimicrobial agents against single-species biofilm of *Prevotella intermedia, Pepto streptococcus micros, Streptococcus intermedius, Fusobacterium nucleatum and Enterococcus faecalis* which are derived from a range of root canal isolates. Biofilms of these species were generated on membrane filter discs and subjected to 15 min or 1 h incubation with 5 p.p.m. colloidal silver, 2.25% sodium hypochlorite (NaOCl), 0.2% Chlorhexidine, 10% Iodine or Phosphate buffered saline (PBS) as a control. The antimicrobial activity was assessed by calculating the Colony Forming Units. They reported that Iodine and NaOCl were more effective than chlorhexidine except against *P. micros* and *P. intermedia* where they were all 100% effective. Iodine and NaOCl elicited a 100% kill after 1 h incubation for all strains used. After 15 min, they showed differing bactericidal effects depending on the strain. None of the agents were effective against *F. nucleatum* after 15 min but NaOCl, iodine and

chlorhexidine were all effective after 1 h. Colloidal silver was generally ineffective. They concluded that NaOCl was generally the most effective agent tested, followed by iodine.

Russell *et al.* 2002[30] evaluated the activity of five simulated antifungal regimens for eradication of catheter-related bloodstream *Candida* infection was evaluated with an *in vitro* pharmacodynamic model. Single-lumen central venous catheters were colonized with *Candida* species by sequentially incubating central venous catheters in plasma and then in growth medium, containing a standardized suspension (10 CFU/ml) of *Candida albicans, Candida glabrata,* or slime- producing *Candida parapsilosis*. Colonized central venous catheters were then placed in a one-compartment pharmacodynamic model where five antifungal regimens (plus control) were simulated: Amphotericin B, 1.0 mg/kg every 24 h; Amphotericin B, 0.5 mg/kg every 24 h; Fluconazole, 400 mg every 24 h; Fluconazole, 800 mg every 24 h; and Voriconazole, 4 mg/kg every 12 h. During exposure to the simulated clinical regimens, samples were serially removed from the model over 48 h for quantitation of viable organisms. They concluded that no regimen, however, completely eradicated (by culture and electron microscopy) central venous catheter colonization. Regrowth was noted in the model during therapy against *C. glabrata* and *C. parapsilosis* but was not associated with an increase in the MICs for the isolates. Lack of *in vitro* antifungal activity against biofilm-encased organisms appeared to be the primary reason for mycological failure of antifungal regimens in the model.

Ferguson *et al.* 2002[31] in an *in vitro* study evaluated the susceptibility of *Candida albicans* to various irrigants and medicaments. The minimum inhibitory concentrations of NaOCl, hydrogen peroxide, chlorhexidine digluconate and aqueous calcium hydroxide were determined. The results revealed that sodium hypochlorite,

hydrogen peroxide and chlorhexidine digluconate were effective against *Candida albicans* even when significantly diluted.

J. C. Yamashita *et al.* 2003[32] evaluated *in vitro* the cleaning of root canal walls after irrigation with saline, 2% CHX, 2.5% NaOCl, 2.5% NaOCl +EDTA. The cleaning of the apical, middle and coronal thirds of the root canals was evaluated by scanning electron microscopy. The best cleaning was obtained using 2.5% NaOCl and EDTA followed by 2.5% NaOCl, whose cleaning was similar to CHX only in the cervical third. Better cleaning was found in the cervical and middle third for all groups with the worst result in the apical third.

C.E. Radcliffe *et al.* 2004[33] evaluated the antimicrobial activity of varying concentrations of NaOCl such as 0.5%, 1%, 2.5%, 5.25% against endodontic microorganisms *A. israelii, A. naeslundii, C.albicans, E. faecalis*. Contact time used was 0, 10, 20, 30, 60 and 120s. In case of *E. faecalis*, additional contact time such as 1.0, 2.0, 5.0, 10.0 and 30mts were tested. Pour plates were used to count low CFU and serial dilutions were used to find high CFU. All concentrations of NaOCl lowered the CFU below the limit of detection after 10s in case of *A. naesulundii* and *C.albicans*. *E. faecalis* proved significantly more resistant to NaOCl. But at higher concentrations less time was required though 5.25% NaOCl was not completely effective after 1 minute.

Mahomed *et al.* 2004[34] evaluated fungicidal activity of Fluconazole on *Candida albicans* under *in vitro* conditions resembling the vaginal microenvironment, using vagina-simulative medium (VS). They found that Fluconazole was fungicidal for *C.albicans* in VS, but not in other media at the same pH, 4.2. In VS, Fluconazole was fungicidal at concentrations of ≥8µg/ml and reduced viability by greater than 99.9%.

Barnhart *et al.* 2005[35] evaluated the cytotoxicity of NaOCl, IKI, Betadine scrub, $Ca(OH)_2$, chlorine dioxide on cultured gingival fibroblast using the CY Quant assay. Human gingival fibroblasts were grown in Dulbeccos modified eagle medium containing 10% foetal bovine serum at 37°C and 5% CO2. Cells were split plated for 24h to allow attachment. Irrigants were tested at different concentrations. IKI and $Ca(OH)_2$ were well tolerated by human gingival fibroblast.

Pfaller *et al.* 2006[36] assessed the developing interpretive breakpoints for Fluconazole and *Candida spp*. These breakpoints were considered to be somewhat weak, because the clinical data supporting them came largely from mucosal infections and there were very few infections involving strains with elevated Fluconazole MICs. The MIC distribution for Fluconazole was determined with a collection of 13,338 clinical isolates. The overall MIC at which 90% of the isolates were inhibited and 3% were resistant .Similar results were obtained for 2,190 isolates from randomized clinical trials.

Sena *et al.* 2006[37] investigated the antimicrobial activity of 2.5% and 5.25% sodium hypochlorite and 2.0% chlorhexidine gel and liquid as endodontic-irrigating substances against selected single-species biofilms of *Enterococcus faecalis, Staphylococcus aureus, Candida albicans, Prevotella intermedia, Porphyromonas gingivalis, Porphyromonas endodontalis and Fusobacterium nucleatum*. The biofilms were then immersed in the endodontic-irrigating substances for 30 s and also for 5, 10, 15, 30 and 60 min, with and without mechanical agitation. They concluded that mechanical agitation promoted the effectiveness of the antimicrobial agents, resulting in less time to eliminate the same micro-organisms, except for *S. aureus* with 2.5% NaOCl. Antimicrobial agents in liquid presentation, especially 5.25% NaOCl and 2% chlorhexidine, killed the tested micro-organisms more rapidly. Saline did not

inhibit the growth of any of the tested micro-organisms, with or without agitation, being statistically different from NaOCl and Chlorhexidine. *P. intermedia, P. gingivalis, P. endodontalis* and *F. nucleatum* were eliminated in 30 s by all antimicrobial agents, with our without agitation, in contrast with the facultative and aerobe strains.

Eweis *et al.* 2006[38] in an *in vitro* study investigated the antifungal behaviour of chitosan and its thiourea derivative on the mycelial growth, sporulation and germination of conidia or sclerotia of the sugar-beet such as: Beta vulgaris pathogens isolated in Egypt, *RhizoctoniasolaniKühn* (AG(2-2)) *Sclerotiumrolfsii Sacc*. and *Fusariumsolani* (Mart.) Sacc. They reported that all the prepared thiourea derivatives had a significant inhibiting effect on the different stages of development on the germination of conidia or sclerotia of all the investigated fungi. In the absence of chitosan and its derivative, *R. solani* exhibited the fastest growth of the fungi studied.

Clegg *et al.* 2006[39] assessed the effectiveness of different concentrations of Sodium hypochlorite (NaOCl), 2% Chlorhexidine (CHX) and BioPure MTAD. Intracanal contents were collected from 10 patients diagnosed with chronic apical periodontitis. The samples were cultured on hemisections of root apices to generate a polymicrobial biofilm. Each biofilm was separately immersed in 6% NaOCl, 3% NaOCl, 1% NaOCl, 2% CHX, 1% NaOCl followed by BioPure MTAD, and sterile phosphate buffered solution (PBS). SEM analysis showed 6% NaOCl and 3% NaOCl were capable of disrupting and removing the biofilm; 1% NaOCl and 1% NaOCl followed by MTAD were capable of disrupting the biofilm, but not eliminating bacteria; 2% CHX was not capable of disrupting the biofilm. Viable bacteria could not be cultured from specimens exposed to 6% NaOCl, 2 % CHX, or 1% NaOCl followed by BioPure MTAD. These results indicate that 6% NaOCl was

the only irrigant capable of both rendering bacteria nonviable and physically removing the biofilm.

Ruff *et al.* 2006[40] investigated the antifungal efficacy of 6%NaOCl, 2%CHX, 17%EDTA and Biopure MTAD as final rinse on *C.albicans*. The specimens were randomly divided into four groups. The experimental teeth were inoculated with *C.albicans* and incubated for 72 hours. The groups were rinsed as follows: 1ml of 6% NaOCl, 0.2% CHX, 1ml of 17% EDTA and 5ml of Biopure MTAD. Aliquots are collected and plated on Sabouraud 4% dextrose agar plates and CFU were counted and they concluded that 2%CHX and 6% NaOCl were equally effective and statistically superior to 17% EDTA and Biopure MTAD.

Elka *et al.* 2007[41] in an *in vitro* study evaluated the effectiveness of the intracanal irrigants such as 17% EDTA, 2% CHX (chlorhexidine),6% NaOCl (sodium hypochlorite), 3% NaOCl and 3% H_2O_2(hydrogen peroxide) used in eliminating *Candida albicans*. They reported that the effectiveness of NaOCl against *Candida albicans* is reduced significantly. The 2% chlorhexidine solution is more effective than 3% NaOCl in eliminating *Candida albicans*.

Monika Marending *et al*. 2007[42] evaluated the impact of different irrigation sequence of 2.5% NaOCl (exposure time 24mts) and 17% EDTA (exposure time 3mts) on the elastic modulus and flexure strength of standardized human root dentin bars prepared from extracted upper third molar. Specimens after exposure to irrigants were subjected to 3- point bending test using a universal testing machine. Modulus of elasticity and flexural strength compared with water or EDTA treated groups whereas the elastic modulus remains unaffected. Short exposure to EDTA did not affect the mechanical dentin parameters.

Gillian *et al.* 2007[43]reviewed the polyene antibiotic Amphotericin B (AMB) which is one of the first therapeutic agents to be marketed commercially as nanosized formulations in which the drug is associated with lipids as liposomes or complexes. In this way, its renal toxicity is reduced and its therapeutic index improved.

Siqueira *et al.* 2007[44]conducted a clinical study to assess the microbial efficacy of 2.5% NaOCl as irrigant, Calcium hydroxide as intracanal medicament. It was concluded that in all the treatment regimens used i.e. irrigation with 2.5% NaOCl and after seven day Calcium hydroxide medicament bacteria and their toxins were reduced and there were no resistant species.

Schafer *et al.* 2007[45]in a review article about action and interactions of root canal irrigants and different irrigation protocols that could be used clinically. Example 2- 5ml. of Sodium hypochlorite between instruments, 5-10ml. of sodium hypochlorite and 5ml. of EDTA for a minute after cleaning and shaping. The use of atleast two irrigating solutions has been advocated. NaOCl dissolves organic components of dentin, pulpal remnants. 17% EDTA can remove the smear layer effectively. Combination of the above i.e. 5.25% NaOCl and 17% EDTA is recommended.

Kishen *et al.* 2008[46] investigate the antibacterial and antibiofilm efficacy of cationic nanoparticulates for root canal disinfection. Experiments were performed in two stages. In stage 1, experiments were conducted to examine the physical properties of three types of nanoparticulates. The antibacterial properties of nanoparticulates alone and nanoparticulates mixed with zinc oxide– eugenol– based sealer were studied. In stage 2, the ability of nanoparticulates-treated dentin to prevent bacterial adherence was examined. Zinc oxide nanoparticulates, chitosan nanoparticulates, a mixture of zinc oxide and chitosan nanoparticulates, and zinc oxide nanoparticulates with multilayered coating of chitosan were tested. This study showed that the

incorporation of nanoparticulates did not alter the flow characteristics of sealer but improved the direct antibacterial property and the ability to leach out antibacterial components. There was a significant reduction in the adherence of *Enterococcus faecalis* to nanoparticulates- treated dentin.

Mohammadi *et al.* 2008[47] in a review about Sodium hypochlorite talks about the history of chlorine releasing agents, mechanism of action, its efficacy as antibacterial, antifungal agent, biofilm removal quoting both *in vivo* and *in vitro* studies. It's tissue solubility, decontamination of operating field, effect on instruments. Sodium hypochlorite may affect the microhardness of dentin by degradation of it's organic components. Bonding to dentin is also adversely affected. It has a good haemostatic property. It's limitations are that it is toxic, damage clothing, eyes, has adverse effects when pushed beyond the foramen (Hypochlorite accident) and allergic reactions. But despite its limitations there is no replacement for sodium hypochlorite as an irrigant in endodontics.

Vytaute *et al.* 2008[48] reviewed that the infected root canal system is a unique niche for the selective species of microorganisms. Certain species of microorganisms especially, Gram-positive facultatives, which often have expanded representation in retreatment cases in comparison with primary endodontic treatment, possess greater resistance to antimicrobial agents used during endodontic treatment than anaerobes. Microbes in the root canals can grow not only as planktonic cells or in aggregates, co-aggregates, but they can also form biofilms consisting of a complex network of different microorganisms. Biofilms are composed of microcolonies of bacterial cells that are distributed in a matrix which consists of exopolysaccharides, proteins, salts and cell material in an aqueous solution. The matrix takes about 85% of the volume of

a biofilm. Bacterial biofilms are reported to be the most common cause of persistent inflammation.

Bryce *et al.* 2009[49]investigated the relative disruption and bactericidal effects of root canal irrigants on single- and dual-species biofilms of root canal isolates. Biofilms of *Streptococcus sanguinis, Enterococcus faecalis, Fusobacteium nucleatum, and Porphyromonas gingivalis* were grown on nitrocellulose membranes for 72 hours and immersed in NaOCl, EDTA, chlorhexidine, and iodine for 1, 5, or 10 minutes. The number of viable and nonviable bacteria disrupted from the biofilm and those remaining adherent were determined by using a viability stain in conjunction with fluorescence microscopy. They reported the most effective agent at disrupting biofilms was NaOCl. Iodine was generally effective at bacterial killing but not disruption.

Mohammad *et al.* 2009[50]evaluated the effect of 17% EDTA and MTAD, both followed by irrigation with 5.25% NaOCl on intracanal smear layer removal, using scanning electron microscopy. They concluded that MTAD is an effective final rinse solution for removing the smear layer in canals irrigated with sodium hypochlorite. When 17% EDTA used as final rinse, the smear layer was removed from the middle and coronal thirds of canal preparations, but it was less effective in the apical third of the canals.

Saurabh *et al.* 2010[51] evaluated the antifungal efficacy of 5.25% NaOCl, 2% CHX and 17% EDTA with and without an antifungal agent. The teeth were inoculated with a suspension of *C.albicans* and then the experimental specimens were divided into 2 groups. The irrigant group was divided into three subgroups and irrigated with 5.25% NaOCl, 2% CHX and 17% EDTA respectively. The irrigant with antifungal group was divided into three subgroups and irrigated with 5.25% NaOCl,

2% CHX and 17% EDTA respectively followed by 1% Clotrimazole. Aliquots from the experimental teeth were plated on 4% Sabouraud dextrose agar and colony forming units were evaluated under light microscopy. They concluded that 5.25% NaOCl exhibit superior antifungal efficacy compared to groups with 2% CHX and 17% EDTA alone and 5.25% NaOCl and 2% CHX with clotrimazole showed significantly greater antifungal properties than 17% EDTA with clotrimazole.

Haapasalo *et al.* 2010[52] in a review on endodontic irrigants summarises the goals of irrigation, ideal endodontic irrigants and details on various endodontic irrigating solutions their chemistry, method of action, use, interactions, advantages and disadvantages. Sodium hypochlorite commonly used concentrations are 0.5%- 6%. It effectively dissolves collagen, pulpal remnants and organic contents of dentin. Although it does not remove the smear layer it aids its removal by dissolving the organic components of the smear layer and then EDTA or citric acid can be used. Sodium hypochlorite has a pH of 11. It's antibacterial efficacy and ability to remove biofilm has been proved and quoting various studies it was stated that higher concentrations of sodium hypochlorite was more effective than lower concentrations. Regarding limitations it has an unpleasant taste and smell, toxicity, cannot remove smear layer by itself. Poorer *in vivo* performance as compared to *in vitro* could be attributed to complexities of the root canal system. Long term exposure to sodium hypochlorite adversely affects the dentin elasticity and flexural strength.

Retamozo *et al.* 2010[53]conducted an *in vitro* study to determine the concentration and time required for sodium hypochlorite irrigation to eliminate *Enterococcus faecalis*. According to this study, the most effective regimen was 5.25% sodium hypochlorite for forty minutes to remove *E. faecalis* contaminated within the root canal dentin.

Shrestha *et al.* 2010[54] evaluated the efficacy of CS-np and ZnO-np in disinfecting and disrupting biofilm bacteria and the long-term efficacy of these nanoparticulates following aging. *Enterococcus faecalis* in planktonic and biofilm forms were treated with different concentrations of CS-np and ZnO-np. The effect of aging by using sterile saliva and phosphate-buffered saline on the antibacterial properties of the nanoparticulates was also determined. They reported the reduction in the thickness of biofilm after nanoparticulate treatment and both CS-np and ZnO-np were found to retain their antibacterial properties after aging for 90 days.

VahidZand *et al.* 2010[55]compared the efficacy of gel and solution forms of NaOCl in removal of smear layer from root canals. The canals of all teeth were prepared with rotary RACE instruments and flushed with 2.5% NaOCl solution and in NaOCl gel group the canals were coated with gel and final rinse with 1ml of 17% EDTA for 2 min. The amount of smear layer was quantified according to the Torabinejad method using SEM. No significant difference between NaOCl solution and gel but there was significant difference between saline and NaOCl gel in the apical, coronal and middle third. Use of NaOCl gel can be effective as NaOCl solution along with EDTA in smear layer removal.

Chavez *et al.* 2010[56] tested the effect of antimicrobials and alkali on biofilms of *Enterococcus faecalis, Lactobacillus paracasei, Streptococcus anginosus,* and *Streptococcus gordonii* isolated from root canals with persistent infections. They reported that NaOCl (1%) affected the membrane integrity of all organisms and removed most biofilm cells. Exposure to EDTA (50 mmol/L) affected the membrane integrity in all organisms but failed to remove more than a few cells in biofilms of *E. faecalis, L. paracasei, and S. anginosus*. Chlorhexidine (2.5%) had a mild effect on the membrane integrity of *E. faecalis* and removed only 50% of its biofilm cells. The

effects were substratum-dependent, and most organisms displayed increased resistance to the antimicrobials on collagen-coated surfaces.

Alburquenque *et al.* 2010[57] in an *in vitro* study evaluated the antifungal activity of low molecular weight chitosan and potential synergy between chitosan and a currently used antifungal drug, fluconazole. The MIC of chitosan and fluconazole against 105 clinical isolates were measured by broth microdilution method. Low molecular weight chitosan exhibited a significant antifungal activity, inhibiting over 89.9% of clinical isolates examined. A greater antifungal activity of low molecular weight chitosan was observed at pH 4.0. There was no evidence of a synergistic effect of the combination of low molecular weight chitosan and fluconazole at pH 7.0.

Luis *et al.* 2010[58] evaluated the antibiofilm and antifungal efficacy of chitosan against *candidal* biofilms, using an *in vivo* central venous catheter model. Confocal and scanning electron microscopic examination demonstrated that chitosan penetrates *candidal* biofilms and damages fungal cells. The concentrations of chitosan that were used to evaluate fungal biofilm susceptibility were not toxic to human endothelial cells.

Stojicic *et al.* 2010[59] evaluated and compared the effects of concentration, temperature and agitation on the tissue dissolving ability of NaOCl. A hypochlorite product with added surface active agent was compared with conventional hypochlorite solution. Thus NaOCl solutions in concentrations of 1%, 2%, 4% and 5.8% were tested at room temperature at 37°C and 45°C on bovine muscle tissue with and without agitation by ultrasonic and sonic energy and pipetting. Percentages of weight loss of tissue specimen were calculated before and after treatment and contact angle on dentin at concentration of 1% and 5.8%. Weight loss of the tissue increased with the concentration of NaOCl. The effect of agitation on tissue dissolution was

greater than that of temperature; continuous agitation resulted in fastest tissue dissolution. Hypochlorite with added surface active agent had the lowest contact angle on dentin.

Rocas *et al.* 2010[60]studied the bacteria in endodontic treatment procedure by using a combined ribosomal RNA-based reverse transcriptase polymerase chain reaction and reverse capture checker board hybridization approach. Samples were taken from infected canals of teeth with apical periodontitis before treatment and after chemicomechanical preparation with NaOCl and after medication with $Ca(OH)_2$ paste. Presence of bacteria was screened by DNA based single PCR assay. RNA extracts were subjected to RT-PCR and were surveyed for the presence of 28 targeted bacteria by checker board method. Bacteria were found in all sample, detectable level of ribosomal RNA was seen in 60% of cases after Chemomechanical preparation and 53% after intracanal medication.

Jaju *et al.* 2011[61] in a review article on endodontic irrigants and newer irrigants has concluded that there is no replacement for sodium hypochlorite as a root canal irrigant in endodontics till date. Despite the limitations of sodium hypochlorite the newer irrigants can be used as an adjunct to sodium hypochlorite not a replacement.

Monteiro *et al.*, 2011[62] evaluated the effect of silver nanoparticles (SN) against *C.albicans* and *C. glabrata* adhered cells and biofilms. MIC results showed that SN were fungicidal against all strains tested at very low concentrations (0.4-3.3 µg ml-1). SN were more effective in reducing biofilm biomass when applied to adhered cells (2h) than to pre-formed biofilms (48h), with the exception of *C.glabrata* ATCC, which in both cases showed a reduction of ~90%.

Kanikireddy *et al.* 2011[63] reviewed the in situ fabrication of chitosan-poly (vinyl alcohol)-silver nanocomposite films in view of their increasing applications as antimicrobial packaging, wound dressing and antibacterial materials. The reduction of silver ions into silver nanoparticles (AgNPs) is achieved in acidic solution of chitosan (C) and poly (vinyl alcohol) (PVA) using their functional groups (-OH, -COOH, $-NH_2$ groups). The anti-microbial and anti-fungal activity of the chitosan- PVA silver nanoparticle films have demonstrated significant effects against *E. coli*, *Pseudomonas*, *Staphylococcus*, *Micrococcus*, *Candida albicans*, and *P. aeruginosa*. To improve further their therapeutic efficacy as anti-microbial agents, curcumin encapsulated chitosan-PVA silver nanocomposite films are developed which showed enormous growth inhibition of *E. coli* compared to curcumin and chitosan-PVA silver nanoparticles film alone.

Priya *et al.* 2011[64] examined the effect of continuous perfusion with antifungals on *Candida albicans* biofilms under conditions of flow, closely mimicking physiological conditions encountered within patients. Biofilms displayed high levels of resistance to fluconazole, and this antifungal exerted minor effect on dispersion levels. Amphotericin B proved effective in reducing viability of cells within the biofilms and dispersion, but only at high concentrations. Under flow conditions, caspofungin exhibited potent activity against biofilms and drastically reduced biofilm dispersion.

Hegde *et al.* 2012[65]in an *in vitro* study on the amount of sodium thiosulphate that was required to neutralize the effect of various concentrations of sodium hypochlorite demonstrated that 1.4ml, 2.4ml and 3.5ml of 5% sodium thiosulphate is required to neutralize 2%, 3% and 5% of sodium hypochlorite respectively.

Moghadas *et al.* 2012[66] evaluated the antimicrobial efficacy of new nanobased irrigant (silver nanoparticles) in comparison with 5.25% NaOCl against *Enterococcus faecalis* and *Staphylococcus aureus* was studied in different time intervals of 3,5 and 15 minutes. They concluded that the new silver nanoparticle based irrigant is as effective as NaOCl in preventing the bacterial growth of common root canal bacteria.

Marion *et al.* 2012[67] in a literature review on the ideal concentration of Sodium hypochlorite used as an endodontic irrigant stated that higher the concentration of sodium hypochlorite used, the greater the toxicity and irritation of periapical tissues but lower concentrations like 0.5% and 1% needs more time to dissolve tissue and organic debris.

Sofi *et al.* 2012[68] tested Starch coated nanosilver for their antibacterial activity against various microorganisms that are commonly found in endodontic failures such as *Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, Acinetobacterbaumani, Candida albicans, Klebsiella pneumonia*. They reported that the synthesized starch coated nanosilver showed good bactericidal effect against a wide range of organisms. The efficacy study using human tooth model shows that there was a significant reduction in the adherence of *Enterococcus faecalis* to nanoparticulates-treated dentin.

Neda *et al.* 2012[69]evaluated anti-candidal effects of Iranian and Korean made injectable calcium hydroxide on *Candida albicans*. They reported the inhibitory effect of Iranian and Korean Calcium hydroxide on *C. albicans*, up to 24 hours is within low range. Higher concentrations of base calcium hydroxide, showed greater inhibition zone on *C. albicans*.

Aysin*et al.* 2012[70] in an *in vitro* study evaluated the antimicrobial activity of 4 antibiotic agents (for *E.faecalis*) and 4 antifungal agents (for *C.albicans*) by agar dilution method. Additionally, modified strip diffusion method was used for detection of *in vitro* antimicrobial activities of 5% NaOCl, 2.5% NaOCl, 17% EDTA and 2% CHX. For intracanal medication, $Ca(OH)_2$-CHX worked efficiently in killing *E.faecalis* isolates compared to $Ca(OH)_2$-Sterile saline solution, $Ca(OH)_2$-Glycerin. For *C.albicans*, 18 isolates were susceptible to Amphotericin B, Nystatin, Fluconazole but showed resistance to Ketoconazole. 5% NaOCl was more effective in eliminating and produced larger inhibition zone compared to 2.5% NaOCl, 17% EDTA and 2% CHX. $Ca(OH)_2$-Glycerin intracanal medication was better in eliminating *C.albicans* isolates and produced larger inhibition zone compared to other $Ca(OH)_2$ medicaments.

Gary *et al.* 2012[71] evaluated the effect of 2.5% sodium hypochlorite and four other intra-canal medications on *Candida albicans* harvested from root canals. The contaminated canals were irrigated with sterile saline and then treated with either, calcium hydroxide + saline; calcium hydroxide + 2.0% chlorhexidine gluconate; zinc oxide + 2.0% chlorhexidine gluconate; Amphotericin B powder + distilled water, or 2.5% sodium hypochlorite. They reported that irrigation with 2.5% sodium hypochlorite was effective in 90% of the samples, calcium Hydroxide + saline was the least effective (50% effective).

Kaya *et al.* 2012[72] evaluated the antifungal activity of a model antifungal (Fluconazole) against *Candida albicans* and *Candida glabrata* strains using a microbroth kinetic assay based on continuous monitoring of changes in the optical density of fungal growth. The minimum inhibitory concentration values for

Fluconazole were determined as 0.31µg/ml for *C.albicans* and 16.32µg/ml. for *C.glabrata.*

Wadhwani *et al.* 2012[73] *in vitro* study evaluated the antifungal efficacy of 5.25% sodium hypochlorite, 2% chlorhexidine gluconate, and 17% EDTA as final irrigant with and without the inclusion of an antifungal agent that is 1% clotrimazole on *Candida albicans*. They concluded that 5.25% sodium hypochlorite exhibited superior antifungal efficacy compared to 2% chlorhexidine gluconate and 17% EDTA. On inclusion of 1% clotrimazole, there was a significant decrease in colony forming units. 5.25% sodium hypochlorite and 2% chlorhexidine gluconate with clotrimazole showed significantly greater antifungal properties than 17% EDTA with clotrimazole.

Pawan *et al.* 2012[74] in an *in vitro* study evaluated the antifungal activity of silver/ Chitosan nanoformulations against important seed borne pathogens. Differences were observed in the antifungal activity of the silver nanoparticle, chitosan nanoparticles and silver/ chitosan nanocomposite, upon the mycelial growth and zone of inhibition of the fungi. Tests for the fungal growth revealed that the nanoformulations showed significant inhibition effectiveness.

Kangarlou *et al.* 2013[75]compared antimicrobial activity of a new irrigation solution containing silver nanoparticles with that of Sodium hypochlorite and chlorhexidine against *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Candida albicans* with direct culture technique. The diameter of growth inhibition zone was determined for different microbial species and they proved that the greatest antimicrobial activity against microbial species belonged to

Sodium hypochlorite 5.25% and 2.5%. Silver nanoparticle solution had an acceptable antimicrobial activity in comparison to other solutions and its antimicrobial property constantly improved by increased concentration of Ag ions.

Mohammadi *et al.* 2013[76]compared the antifungal activity of 1.3% NaOCl, 2%CHX, MTAD and Tetraclean as final rinse against *C.albicans* in a human tooth model *in vitro*. After preparing the root canals, teeth were inoculated with *C.albicans* and incubated for 72 h. Teeth were divided into four experimental groups according to the irrigation solution: NaOCl, CHX, MTAD and Tetraclean. After culturing aliquots from the experimental teeth on Sabouraud 4% dextrose agar, colony-forming units were counted and then they concluded that 1.3% NaOCl and 2% CHX were equally effective and significantly superior to MTAD and Tetraclean. Furthermore, the antifungal efficacy of tetraclean was significantly superior to MTAD.

Glassman *et al.* 2013[77] in a review on endodontic irrigants and irrigant delivery systems emphasises on the need of irrigation for proper decontamination of the root canal system. Irrigants such as sodium hypochlorite the best in removing endodontic biofilms and can be coupled with other irrigating solutions such as EDTA. But precautions like the use of rubber dam should be taken while using the above. Irrigant delivery systems from manual agitation of the irrigants to machine assisted agitation such as sonics and ultrasonics, as well as newer systems such as EndoVac (Sybron Endo), which delivers apical negative- pressure irrigations, plastic rotary F file (Plastic Endo), Vibringe (vibringe), the Rinsendo (Air Techniques) and the EndoActivator (DENSPLY Tulsa Dental Specialties) have all been discussed.

Afzal *et al.* 2013[78] in an *in vitro* study establishes once again that 5.25% Sodium hypochlorite is the most effective root canal irrigant in eliminating *E. faecalis* biofilm and despite its drawbacks and side effects an alternative to this irrigating solution is yet to be found.

Aashish *et al.* 2013[79] reviewed the influence of root canal irrigants on dental tissues. Irrigation solutions used during endodontic treatment react with organic and inorganic components of dental hard tissues, thereby, altering the physical and chemical properties like adhesion, modulus of elasticity, microhardness, tensile & flexural strength of the tooth thereby predisposing to fracture. The cytotoxicity of these agents is concentration dependent, higher the concentration more is the side-effects. Therefore, to attain maximum benefits of the irrigating solutions only minimal required concentration should be used.

Bruhvi *et al.* 2013[80] investigated twenty symptomatic root‑filled teeth with chronic apical periodontitis for the prevalence of *Enterococcus faecalis* and *Candida albicans* in the root filled teeth associated with symptomatic cases with or without periradicular lesions. They reported that by PCR amplification of the samples using taxon specific primers, *E. faecalis* was found to be prevalent species, detected in 65% of the cases and *C. albicans* was detected in 35% of cases. Geographical influence and dietary factors might have some role to play in the prevalence of the species like *C. albicans* and presence of *E. faecalis* confirming the assertion of previous culture dependent and independent approaches for the microbiological survey of root filled teeth.

Monteiro *et al.* 2013[81] evaluated the antifungal efficacy of SN in combination with Nystatin or chlorhexidine digluconate against *Candida albicans* and *Candida glabrata* biofilms. The drugs alone or combined with SN were applied on mature *Candida* biofilms (48 h), and after 24 h of treatment their antibiofilm activities were assessed by total biomass quantification (by crystal violet staining) and colony forming units enumeration. They concluded that SN combined with either Nystatin or Chlorhexidine digluconate demonstrated synergistic antibiofilm activity, and this activity was dependent on the species and on the drug concentrations used. SEM images showed that some drug combinations were able to disrupt *Candida* biofilms.

Silva *et al.* 2013[82] in an *in vitro* study compared the biofilm formation by *Candida glabrata* and *Candida albicans* on acrylic and then examined the antimicrobial effects of silver nanoparticles and Nystatin on these biofilms. They reported that both species adhered to and formed biofilms on acrylic surfaces and both silver nanoparticles and Nystatin has inhibitory effects on biofilms of these species.

Solmaz *et al.* 2013[83] evaluated the antibacterial properties and characterization of chitosan- silver nanoparticle composite materials. *Escherichia coli, Acinetobacterbaumannii, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa* and *Streptococcus pneumonia* were used to test the bactericidal efficiency of synthesized chitosan-silver nanoparticle composite materials. The biological activity was determined by the minimal bacterial concentration of the materials. Antibacterial effect of chitosan- silver nanoparticle materials was increased by increasing Ag amount of the composite materials. The presence of small amount of metal nanoparticles in the composite was enough to significantly enhance antibacterial activity as compared with pure chitosan.

Shweta *et al.* 2014[84] reviewed the nanoparticle-based drug delivery systems. The use of conventional antimicrobial agents against these infections is always associated with problems such as the development of multiple drug resistance and adverse side effects. In addition, the inefficient traditional drug delivery system results in inadequate therapeutic index, low bioavailability of drugs and many other limitations. Antimicrobial nanoparticles and nanosized drug delivery carriers have emerged as potent effective agents against the infections. Nanoparticles have unique properties owing to their ultra-small and controllable size such as high surface area, enhanced reactivity, and functionalizable structure. Nanoparticles use various antimicrobial mechanisms against the pathogens; they may disrupt the cell membrane directly, or form free radicals. In comparison to the conventional antibiotics, nanostructured antimicrobial agents help in reducing the toxicity, overcoming resistance and lowering the cost. In addition, nanosized drug carriers are also available, which can efficiently administer the antibiotics by improving the therapeutics and pharmacokinetics of the drug.

Saad *et al.* 2014[85] in an *in vitro* study evaluated the ability of *Candida albicans* and *Enterococcus faecalis* to penetrate dentinal tubules of instrumented and retreated root canal surface of split human teeth. The teeth were grouped into 4 groups, negative control, and positive control without canal instrumentation, instrumented, and retreated and then teeth were split longitudinally and inoculated with both microorganisms separately and in combination. Penetration of *C. albicans* and *E. faecalis* into the dentinal tubules was observed in all 3 groups, penetration was partially restricted by dentin debris of tubules in the instrumented group and remnants of canal filling materials in the retreated group. In all 3 groups,

E. faecalis penetrated deeper into the dentinal tubules by way of cell division than *C. albicans* which built colonies and penetrated by means of hyphae. Microorganisms can easily penetrate dentinal tubules of root canals with different appearance based on the microorganism size and status of dentinal tubules.

Sanaa *et al.* 2014[86]analyzed extracellular biosynthesis of silver nanoparticles (Ag-NPs) using *Kluyveromycesmarxianus, Candida utilis* 22 and evaluating the antibacterial and antifungal efficacy against *Staphylococcucaureus* ATCC 6538, *Escherichia coli* ATCC 10536, *Pseudomonas flourescence* ATCC 50090, *Candida albicans, Candida glabrata, Candida krusei* as multi-drug resistant human pathogens. They reported that Ag-NPs have the highest antibacterial and antifungal efficacy against all the tested microorganisms. Silver nanoparticles from each strains have great potential to be an effective to antibacterial and/or antifungal agents for future therapies in multi-drug resistant human pathogens of bacteria and *Candida* infections.

Amanda *et al.* 2014[87] evaluated the effect of silver nanoparticles on *Candida glabrata*, *Candida tropicalis, Staphylococcus aureus*, and *methicillin-resistant Staphylococcus aureus* (MRSA). They found that the AgNPs (1000 μg/mL) promoted reductions in biofilm mass of ~60% for *C. glabrata* and ~35% for *C. tropicalis*. A reduction of ~20% in *C. tropicalis* biomass was also observed at the concentration of 3.91 μg/mL. No significant effect on total biomass was found for *S. aureus* and MRSA. SEM images showed that *C. glabrata* and *C. tropicalis* biofilm cells, exposed to the AgNPs (1000 μg/mL), had an irregular and shriveled appearance. AgNPs solution exhibited considerable antimicrobial activity against important fungal

and bacterial pathogens, associated with several oral and systemic diseases, and has potential as an antimicrobial agent.

Shaik *et al.* 2014[88]examined the sustained release of intracanal medicaments with or without a carrier and tested their antimicrobial efficacy in root canal against *Candida albicans* and *Enterococcus faecalis*. Chitosan was used as vehicle for triple antibiotic paste (TAP) and calcium hydroxide and antimicrobial assessment was performed on second and seventh day. They concluded that Combination of TAP + chitosan and $Ca(OH)_2$ + chitosan produced better results compared with the combination of medicaments with saline.

Yu Pu *et al.* 2014[89] evaluated the *in vitro* damage of *Candida albicans* biofilm by chitosan. Biofilms, a protected niche for microorganisms, are resistant to a range of current antifungal agents. Chitosan, a polyatomic biopolymer with advantageous biocompatibility, biodegradation, nontoxicity and antibacterial properties. As assessed by cell viability assay, chitosan showed significant inhibitory effects on the planktonic cells and biofilms of *Candida albicans* in a dose- dependent manner.

Eduardo *et al.* 2014[90] evaluated the antimicrobial and antibiofilm activity of chitosan to inhibit *C. albicans* growth and biofilm formation. They reported that chitosan is capable of inhibiting *C. albicans* planktonic growth and also inhibited *C. albicans* adhesion biofilm formation and reduced mature biofilms and dual species biofilms (*C. albicans and S. mutans*). So it can be used as an effective anti-Candida agent which is capable of acting on *C. albicans* infections.

Soujanya, *et al.* 2015[91] compared the antimicrobial efficacy of QMixTM 2 in 1, sodium hypochlorite (NaOCl), and chlorhexidine (CHX) against *Enterococcus*

faecalis and *C. albicans*. Samples were divided into two groups. Group I was inoculated with *E. faecalis* and Group II with *C.albicans* and incubated for 3 days. Each group was subdivided into four subgroups based on the type of irrigant used. Group IA, IIA, 5.25% NaOCl; Group IB, IIB, 2% CHX; Group IC, IIC, QMixTM 2 in 1; and Group ID, IID, 0.9% saline (the control group). Aliquots were collected and plated on brain heart infusion agar and Sabouraud dextrose agar. The plates were incubated at 37°C for 24 h and counted the colony forming units (CFUs). They proved that QMixTM 2 in 1 has significant antimicrobial efficacy against *E. faecalis* and *C. albicans.*

Adam *et al.* 2015[92] investigated whether coordination of Cu (II) ions to Fluconazole affects its antifungal activity. The *in vitro* susceptibility tests and antifungal studies were performed with two *Candida spp: Candida glabrata* and *Candida albicans*. Overall, 34 strains of the former and 16 strains of the latter were treated with fluconazole, its Cu (II) complex and free Cu (II) ions. The obtained MIC values in 16 cases of the *C. glabrata* and in 5 cases of the *C. albicans* were lower for the complex in comparison to the drug. This implies that the complex is more effective against particular strains than the parent drug. The most significant improvement in the complex drug efficacy was observed for Fluconazole-resistant species.

Paulo *et al.* 2015[93] in an *in vitro* study determined the susceptibility of oral specimens and ATCC lineages of *Candida albicans* for five endodontic sealers, which were pure and associated with two antifungal drugs and to analyze their effect on the physical properties. They reported that the pure versions of the Sealer 26, AH Plus, Endofill, Fillapex, and Sealapex demonstrated antifungal activity, with Endofill

presenting the greatest inhibition zones. All cements, except for Endofill, had their antifungal actions enhanced by addition of ketoconazole and fluconazole and the AH Plus presented the best antifungal activity. The addition of antifungals to endodontic sealers enhanced the antimicrobial action of most cements tested without altering their physical properties.

MATERIALS & METHODS

Materials used in the study :

a) 3% NaOCl- Freshly Prepared

b) Sodium Azide - Choice Organochem, Mumbai, India.

c) 5.25% NaOCl- Novo Dental Products Pvt Ltd. Mumbai, India.

d) 17% EDTA- Desmear, Anabond Stedman Pharma Research Ltd. Tamilnadu, India.

e) Saline – 0.9% W/V, Baxter (India) Pvt Ltd. Tamilnadu, India.

f) Double Distilled Water- Nice Chemical Laboratory Supplies, Ltd, India.

g) Paper points- Dentsply Maillefer, Switzerland.

h) Type-II GIC- GC Corporation, GC America Inc.

i) ATCC10231 Strain *Candida albicans*- ATCC, Himedia Laboratories Pvt Ltd. Mumbai, India.

j) Sabourose Dextrose Agar (4%)- Himedia Laboratories Pvt Ltd. Mumbai, India.

k) Sabourose Dextrose Broth- Himedia Laboratories Pvt Ltd. Mumbai, India.

l) Petri dishes- Astra Scientific Systems Pvt Ltd. Kerala, India.

m) Glass Test tubes- Lab Tech Medico Pvt Ltd. India.

n) Glass Beaker- Lab Tech Medico Pvt Ltd. India

o) Inoculation Loop- Himedia Laboratories Pvt Ltd. Mumbai, India.

p) Fluconazole- Cipla,Uttarakhand, India.

q) Amphotericin B- United Biotec, Himachal Pradesh, India.

r) Clotrimazole- Denmark, Mumbai, India.

s) Sodium borohydride- Sisco research laboratories Pvt Ltd. Mumbai, India.

t) Silver nitrate- High Purity laboratory chemicals Pvt Ltd. Mumbai, India.

u) Acetic acid- High Purity laboratory chemicals Pvt Ltd. Mumbai, India.

v) Chitosan- Sigma Aldrich, Iceland.

w) Effon drops- Lab tech Medico Pvt Ltd. India.

EQUIPMENTS/ INSTRUMENTS USED IN THE STUDY:

a) Micro motor handpiece- contra angle hand piece, NSK Japan.

b) Micro motor handpiece- straight hand piece, NSK Japan.

c) Barbed Broaches- Mani Inc. Tochigi, Japan

d) K-File- Mani Inc. Tochigi, Japan.

e) Gates glidden drill – Stainless steel, Prime Dental, India.

f) Diamond disc- SS White, USA.

g) Light Microscopy- Olympus Pvt Ltd. Delhi, India.

h) Autoclave- All American 41 Quart Electric Sterilizer Model #75X.

i) Incubator- KEMI.

j) Laminar air flow- Lab Line.

k) Magnetic stirrer- REMI.

METHODOLOGY

PREPARATION OF SAMPLE:

Freshly extracted 50 single rooted mandibular premolar teeth were stored in saline and were used within three months of extraction. Each tooth was digitally radiographed (RVG) in both mesiodistal and buccolingual directions to confirm the presence of a single canal. The teeth were immersed in 3%NaOCl (freshly prepared) for 15 minutes to remove debris and organic tissue. Then teeth were cleaned using an ultrasonic scaler to render them free from calculus and tissue tags. Finally teeth were stored in 0.2% sodium azide (Choice Organochem, Mumbai) until use. The teeth were decoronated at the cementoenamel junction using diamond disc (SS White) and pulpal remnants was extirpated using barbed broaches (Mani). A 25mm size 15 stainless steel K-file (Mani) was inserted into the root canal until it was seen at the apical foramen. Working length was obtained by subtracting one millimeter from this length. Gates Glidden drills 1 to 4 (stainless steel, Prime dental, India) was used for coronal flaring and apical preparation was done until ISO size 50. Between each filing 3mL of 5.25%NaOCl (Novo Dental Products Pvt Ltd, Mumbai) was used. After instrumentation, the removal of smear layer was accomplished with a final rinse of 1mL of 17% EDTA (Anabond stedman) for 1 minute followed by 3mL of 5.25%

NaOCl (Novo dental). Finally, the canals were flushed with 5mL of saline (0.9% w/v, Baxter) to remove any debris and residual irrigants. The roots were coated with two coats of nail varnish and apical foramen sealed with Type II GIC (GC). Subsequently, the roots were sterilized in an autoclave for 15minutes, at 121°C and 15 lb pressure.

Inoculation of fungi into the samples:

A suspension of *Candida albicans* was adjusted to 0.5% turbidity on the McFarland scale (1.5×10^{8}bacteria/mL).The canals of the experimental teeth was cautiously inoculated with 0.5mL of the freshly prepared suspension and were stored in a glass test tube. The samples were stored and incubated at 37°C and 91% humidity for 96 hours. Every 24 hours, the vials containing the experimental teeth were replenished with freshly prepared suspension of *Candida albicans* (ATCC, Himedia Laboratories, Mumbai). After 48 hours, aliquots was taken from each tooth using a syringe and plated on 4% Sabouraud dextrose agar plate to verify the growth of *Candida albicans*. At the end of 96 hours, teeth were removed from the glass test-tube vials. Finally excess fluid in the canal was removed with sterile paper points (Dentsply) and then subjected to the experimental groups of 10 teeth each.

Synthesis of Chitosan-Silver Nanocomposite:

The chitosan suspension was prepared by solubilizing chitosan (1.0 g) in acetic acid (50 mL, 1.0 wt %) solution. Then, AgNO3 (50 mL, 0.01 M) was added immediately into the suspension under constant stirring for 2.0 hours for preparation of the AgNO3 in chitosan suspension. NaBH4 (20 mL, 0.04 M) was added to the suspension of AgNO3/Cts and an immediate color change from pale yellow to brown indicating the formation of Silver nanoparticles was noted.

STUDY GROUPS:

GROUP I -5.25%NaOCl +17%EDTA +0.9% Saline

GROUP II -5.25%NaOCl +17%EDTA +Chitosan-Silver Nanocomposite (20%Ag) + 0.9% Saline

GROUP III -5.25%NaOCl +17%EDTA +0.2%Fluconazole + 0.9% Saline

GROUP IV -5.25%NaOCl +17%EDTA +1% Clotrimazole + 0.9% Saline

GROUP V -5.25%NaOCl +17%EDTA +0.2% Amphotericin B + 0.9% Saline

Irrigation protocol:-

The teeth were randomly divided into 5 experimental groups of 10 teeth each. Samples in Group I (control group) was rinsed with 5.25%NaOCl, 17%EDTA and 0.9% Saline. Group II was rinsed with 5.25%NaOCl, 17%EDTA, Chitosan-Silver Nanocomposite (20%Ag) and 0.9% Saline. Group III was rinsed with 5.25%NaOCl, 17%EDTA, 0.2%Fluconazole (Cipla, Uttarakhand) and 0.9% saline. Group IV was rinsed with 5.25% NaOCl, 17% EDTA, 1% Clotrimazole (Denmark, Mumbai) and 0.9% Saline. Group V was rinsed with 5.25%NaOCl, 17%EDTA, 0.2% Amphotericin B (United Biotec, HP) and 0.9% Saline.

Assessment of antifungal activity:

The time of contact of each irrigant was 1 minute and final flush with 5mL of distilled water. The antifungal agents were injected into the root canal by using a 26 gauge needle. The time of contact was 5 minutes; after which, the sample was flushed with 5mL of distilled water to prevent carryover of irrigants. All experimental teeth were flushed with 15mL of sterile saline (0.9 wt%, Baxter) and canals were dried with sterile absorbent paper points. A small amount of saline solution was introduced into the canal, and then an endodontic hand file was used in a filing motion to a level approximately 1mm short of the root apex. A 1µm inoculation loop (Himedia laboratories Pvt Ltd, Mumbai) was used to remove aliquots from the fluid and was plated on Sabouraud 4% dextrose agar (Himedia Laboratories Pvt Ltd, Mumbai). The plates were incubated at 36°C and 91% humidity for 48 hours. After the incubation period, the growth of *Candida albicans* was assessed with light microscopy at 400X. The number of Colony Forming Units (CFUs) of candida serves as a measure of the antifungal activity.

Statistical analysis:

The data was expressed in MEAN±SD. Statistical Package for Social Sciences (SPSS 16.0) version was used for statistical analysis. One way ANOVA was applied for analysis. Post Hoc followed by Dunnet t test used to find statistical significance between and within the groups. P value less than 0.05 ($P<0.05$) was considered statistically significant at 95% confidence interval.

RESULTS & OBSERVATIONS

Table 1- shows Mean antifungal activity of different groups in which Group I (control), Group II (chitosan –silver nanocomposite) and Group IV (1% clotrimazole) showed the best results with mean and standard deviation of 0.00±0.00 CFU/µl followed by Group III (0.2% Fluconazole) showing Mean ± SD of 0.30±0.48CFU/µl and finally Group V (0.2% Amphotericin B) with least Mean ± SD value of 0.40±0.96 CFU/µl.

Table 2- compares the antifungal activity and P values of Group I (control) with other groups in which $P<0.05$ is significant. Group I with Group II has P value of 1.00 which is not significant. Group I with Group III has P value of 0.02 which is significant. Group I with Group IV has P value of 1.00 which is not significant. Group I with Group V has P value of 0.01 which shows significance.

Table 3 -compares the antifungal activity and P value of Group II (Chitosan-silver nanocomposite) with other groups in which $P<0.05$ is significant. Group II with Group I has P value of 1.00 which is not significant. Group II with Group III has P value of 0.02 which shows significance. Group II with Group IV has P value of 1.00, which is not significant. Group II with Group V has P value of 0.01, which shows significance.

Table 4-compares the antifungal activity and P value of Group III (0.2% Fluconazole) with other groups in which $P<0.05$ is significant. Group III with Group I, Group II and Group IV has P value of 0.02 which shows significance. Group III with Group V has a P value of 0.03 which also shows significance.

Table 5-compares the antifungal activity and P value of Group IV (1% Clotrimazole) with other groups in which P<0.05 is significant. Group IV with Group I and Group II has a P value of 1.00 which is not significant. Group IV with Group III has P value of 0.02 which shows significance. Group IV with Group V has P value of 0.01 which shows significance.

Table 6-compares the antifungal activity and P value of Group-V (0.2% Amphotericin B) with other groups in which P<0.05 is significant. Group V with Group I, Group II and Group IV has P value of 0.01, which shows significance. Group V with Group III has P value of 0.03 which also shows significance.

Table 7 and Graph 1 show multiple comparison of antifungal activity of different groups. Group I, Group II, Group IV showed similar antifungal activity. Group III and Group V showed less significant antifungal activity compared to Group I, Group II and Group IV. 0.2% Fluconazole showed more antifungal activity than 0.2% Amphotericin B.

DISCUSSION

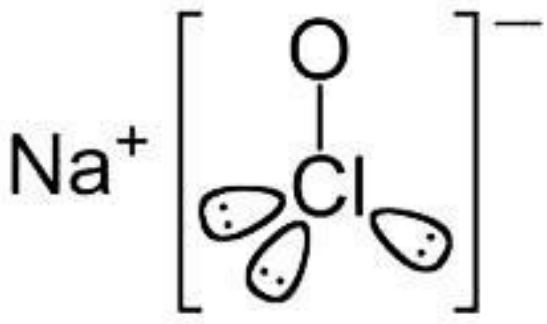
The teeth specimens treated with chitosan- silver nanocomposite showed better antifungal efficacy in inhibition of growth of *C.albicans*. This confirmed the study hypothesis that chitosan- silver nanocomposite has better antifungal efficacy against *C.albicans*.

In 1891, Miller's "Theory of Focal Infection", describes how bacteria roving through the blood stream can cause generalized or localized infection. Thus in the 19th and 20th centuries dentists and physicians advocated the extraction of pulpless and endodontically treated teeth. Kakehashi *et al.* proved that bacteria was the root cause of endodontic disease and therefore advocated that the root canal should be sterilized by eliminating vital and necrosed pulp tissue, bacteria and their toxins. This could be done by thorough instrumentation, irrigation, intracanal medications and finally obturation.[1]

Long term success in endodontic treatment depends on the complete debridement and disinfection of the pulp space. Despite thorough mechanical preparation, pulp remnants, debris and bacteria may be present in the irregularities of root canal system.[94] Hence it is highly desirable that instrumentation should be accompanied by irrigation and intracanal medicaments for the elimination of the microorganisms.[95]Irrigation aims to flush out debris, lubricating the canal and dissolving organic and inorganic tissue. An ideal endodontic irrigant should be germicidal and fungicidal. It should be non-irritating to the periapical tissues. It should have prolonged antimicrobial effect. It should not interfere with the repair of periapical tissues. It should not interfere with the sealing ability of filling materials.[1]

A single irrigant cannot successfully fulfill all the requirements needed during the process of irrigation during root canal treatment. A minimum of two irrigation solutions are required and a specific sequence needs to be followed to optimize the effect of irrigation. Conventional irrigants used during instrumentation are sodium hypochlorite, Ethylene diamine tetra acetic acid, citric acid, chlorhexidine digluconate etc. These are used in various concentrations for proper effective cleaning and disinfection of the root canal system.[52] In this study 5.25% Sodium hypochlorite and 17% EDTA followed by 0.9% normal saline have been used as routine root canal irrigants.

Sodium hypochlorite has been used as an endodontic irrigant since 1920. It is an oxidizing as well as hydrolyzing agent and has bactericidal, virucidal as well as proteolytic properties and has a reasonable shelf life.[21] Root canal irrigation is done with sodium hypochlorite with concentrations ranging from 0.5% to 6% (pH-11). It is an ideal irrigant to remove the organic matter in the pulp chamber including vital as well as necrosed pulp and successful in removing the endodontic biofilm.[52]



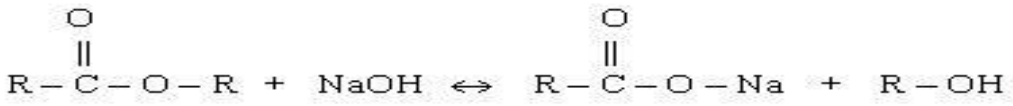
Sodium Hypochlorite Chemical Structure

The mechanism of action of sodium hypochlorite is as follows:

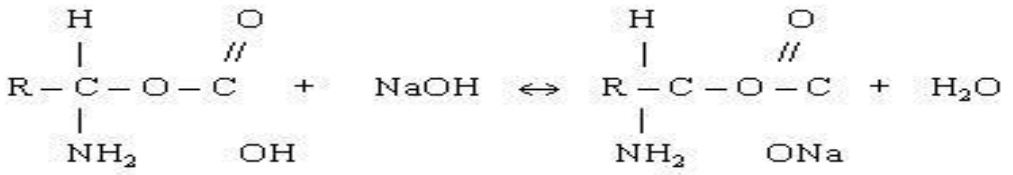
$$NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$$

Sodium hypochlorite works as an organic and fat solvent, degrading fatty acids and transforming them into fatty acid salts (soap) and glycerol (alcohol) and thus reduces the surface tension of the solution (saponification reaction). Next is the amino acid neutralization reaction in which sodium hypochlorite neutralizes amino acids forming water and salt and there is the removal of hydroxyl ions causing a decrease in the pH. The aminoacid neutralization reaction also occurs when hypochlorous acid ($HOCl^-$) present in sodium hypochlorite solution contacts organic tissue and acts as solvent releasing chlorine, which in turn combines with the protein of the amino group to form chloramines. The hypochlorous acid and hypochlorite ions (OCl^-) cause amino acid degradation and hydrolysis. The chloramination reaction between chlorine and the amino group (NH^+) leads to the formation of chloramines that interfere in cell metabolism. Chlorine is a strong oxidizing agent that has an antimicrobial action, inhibiting bacterial enzymes and toxins leading to an irreversible oxidation of sulphydryl group (SH groups) of essential bacterial enzymes. Thus, the saponification, amino acid neutralization and chloramination reactions that occur in the presence of microorganisms and organic tissue lead to the antimicrobial effect and tissue dissolution process.[21,11,47]The amount of active chlorine is <10%.

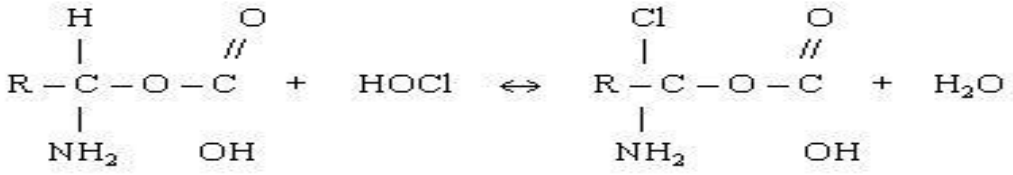
Scheme 1. Saponification reaction.



Scheme 2. Amino acid neutralization reaction.



Scheme 3. Chloramination reaction.



Studies prove that 5.25% NaOCl has been widely used for many years and it prevails as the golden standard today.[96,11] Interaction of NaOCl with the tissue fluids, blood, dentin and other organic debris can reduce the effectiveness. Chemomechanical preparation is a short term procedure and NaOCl remains in the canal for only a few minutes. So the antimicrobial effectiveness of NaOCl within the root canal is a function of concentration and contact time.[97]Studies regarding the concentration of NaOCl ranging from 0.5% to 5.25% has been conducted in the past. Concerns regarding cytotoxicity dependent concentration of NaOCl have led Bystrom and Sundqvist recommend 0.5% NaOCl as the ideal concentration with antimicrobial

action.[95]But this contradicts the study conducted by Vianna *et al.* which conclude that reduction in the concentration of 5.25% NaOCl decreased antimicrobial effectiveness against the anaerobes tested.[98] In the present study a contact time of 5 minute was taken as the standard time for all the irrigants. This could be explained on the basis of maximum antimicrobial action exhibited by chitosan-silver nanocomposite and 1% Clotrimazole in a 5 minute contact time during the pilot study. Results from previous studies have shown that 5.25% NaOCl can eliminate *C.albicans* in a short exposure time of 30 seconds and 2 minute[99,100] which contradict the findings of the present study. The difference in contact time may be attributed to the following factors.

1. In the previous study there is direct contact of microorganism with the antimicrobial agent, fungal suspension were mixed with the antimicrobial agent where as in the present study the dentinal tubules are inoculated with the organism to simulate the clinical condition.

2. The inhibitory effect of dentin on the antifungal effect of the irrigant has been documented.[101]

During root canal instrumentation, dentin chips along with organic debris and irrigating solution forms a smear layer which primarily consists of two layers:

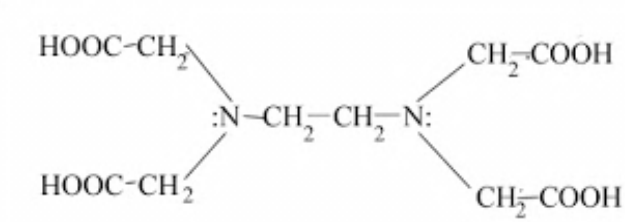
1) 1-2µm of thick layer of organic matter and dentin particles.

2) Extending into dentinal tubules to a depth of 40µm (smear plugs) which is formed largely of dentine chips.

Sodium hypochlorite also has its demerits when used as a root canal irrigant. Hypochlorite solutions are said to adversely affect the mechanical properties of dentin

due to dissolution of the dentinal organic components. NaOCl can cause a concentration-dependent reduction in the elastic modulus and flexural strength in human root canal dentin. This could reduce the carbon and nitrogen content of the dentin matrix. Furthermore dissolution of dentinal collagen causes degradation of dentin.[47]

Neutral Ethylene diamine tetraacetic acid (EDTA) solutions in concentrations ranging from 15-17%, are effective in demineralizing dentin and can be used to remove the smear layer but cannot remove organic debris when used as a sole irrigant and when used in combination with sodium hypochlorite effectively removes organic as well as inorganic debris.[102]EDTA, chelating agent was introduced by Nygaard- Østby. It reacts with the calcium ions in dentin to form soluble calcium chelates. EDTA contains four acetic acid groups attached to Ethylenediamine.[7]



structure of EDTA

EDTA is relatively nontoxic and only a slight irritant in weak solutions. It forms highly stable, soluble, metal chelates in combination with heavy metals or alkaline earth ions. Because it is not metabolized, EDTA may be used to remove calcium from the body, to form calcium chelate. Salts of EDTA may be used to chelate the calcium ions of tooth structure and so decalcify dentin. Studies have reported that EDTA can demineralize dentin upto a depth of 20-30µm in five

minutes.[103] Irrigation with 17% EDTA enhances the permeability of dentin enabling deeper dentinal tubular penetration of root canal irrigants.[77]

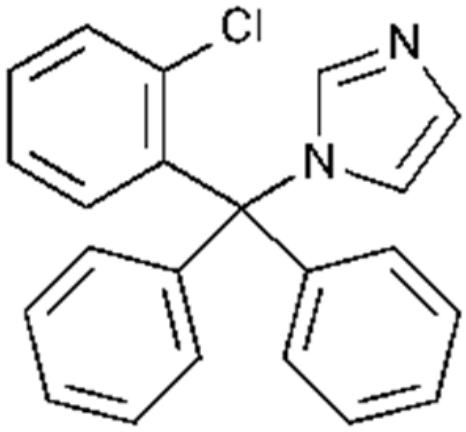
Therefore a combination of 5.25% NaOCl and 17%EDTA was the choice of irrigants used in this study which would enable the better elimination of microorganisms. Interaction between EDTA and NaOCl shows some effects, EDTA retains its calcium complexing ability when mixed with NaOCl, but EDTA causes NaOCl to lose its tissue- dissolving capacity. Therefore EDTA and NaOCl should never be mixed. After irrigation of the canals with EDTA, 2ml of NaOCl should be finally used to neutralize the acidic effects of EDTA and to allow NaOCl to penetrate into dentinal tubules, which are opened after the use of EDTA. No single irrigant is capable of dissolving the organic pulpal material and pre-dentin as well as demineralizing the inorganic calcified portion of the canal wall. Instead, combination of EDTA and NaOCl was proved to be effective in removing smear layer completely.[77]

0.9% Normal saline used as final irrigant in the present study. Normal saline causes gross debridement and lubrication of the root canals. Since it is very mild in action, it can be used as adjunct to chemical irrigants. It basically acts by flushing action. Normal saline used as final rinse for root canals to remove any chemical irrigant left after root canal preparation.[11]

Even after thorough chemo-mechanical preparation, microbes can still be recovered from canals. To combat the emerging antimicrobial resistance and considering the undesirable side effects of synthetic drugs, nanoformulations might

prove to be advantageous. Approximately 60 to 80% of the world population relies on traditional medicines for treatment of common illness.[105]

Clotrimazole, an imidazole derivative, is primarily used locally in the treatment of vaginal and skin infections due to yeasts and dermatophytes. Clotrimazole works to kill individual candida or fungal cells by altering the permeability of fungal cell wall. It binds to phospholipids in the cell membrane and inhibits the biosynthesis of ergosterol and other sterols required for cell membrane production. This leads to the cell's death via loss of intracellular elements. Clotrimazole has been used successfully in patients who had failed to respond to other antifungal agents such as Nystatin and Amphotericin B.[51]

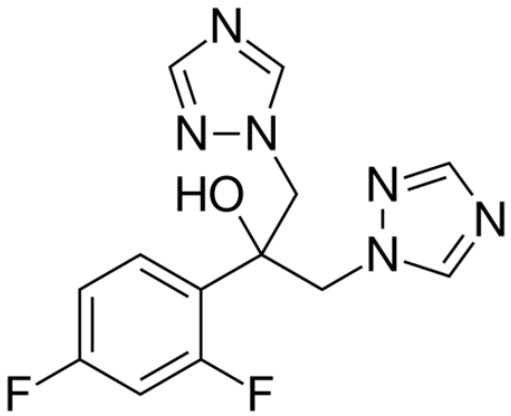


Clotrimazole is an odorless, white crystalline substance. It is practically insoluble in water, sparingly soluble in ether and very soluble in propylene glycol 400, ethanol and chloroform. Clotrimazole is a broad spectrum antifungal agent that is used for the treatment of dermal infections caused by various species of pathogenic dermatophytes and yeasts. The primary action of Clotrimazole is against dividing and growing organisms. *In vitro,* Clotrimazole exhibits fungistatic and fungicidal activity

against isolates of *Trichophyton rubrum, Trichophyton mentagrophytes, Epidermophyton floccosum, Microsporum canis,* and *Candida species,* including *Candida albicans*. Strains of fungi having a natural resistance to Clotrimazole are rare. Only a single isolate of *Candida guilliermondi* has been reported to have primary resistance to Clotrimazole. No single step or multiple step resistance to Clotrimazole has developed during successive passages of *Candida albicans* and *Trichophyton mentagrophytes*. No appreciable change in sensitivity was detected after successive passages of isolates of *C. albicans, C. krusei, or C. pseudo tropicalis* in liquid or solid media containing Clotrimazole. Also, resistance could not be developed in chemically induced mutant strains of polyene resistant isolates of *C. albicans*. Slight, reversible resistance was noted in three isolates of *C. albicans* tested by one investigator. There is a single report that records the clinical emergence of a *C. albicans* strain with considerable resistance to flucytosine and miconazole, and with cross resistance to Clotrimazole; the strain remained sensitive to Nystatin and Amphotericin B. In studies of the mechanism of action, the minimum fungicidal concentration of Clotrimazole caused leakage of intracellular phosphorus compounds into the ambient medium with concomitant breakdown of cellular nucleic acids and accelerated potassium efflux. Both these events began rapidly and extensively after addition of the drug. The imidazoles are preferred over other antifungal drugs because of their greater efficacy, fewer side effects and lesser drug interactions. 1% Clotrimazole showed better antifungal efficacy in previous studies when used in combination with endodontic irrigants.[51]

Fluconazole is a first-generation triazole antifungal medication. It differs from earlier azole antifungals (such as ketoconazole) in that its structure contains a triazole

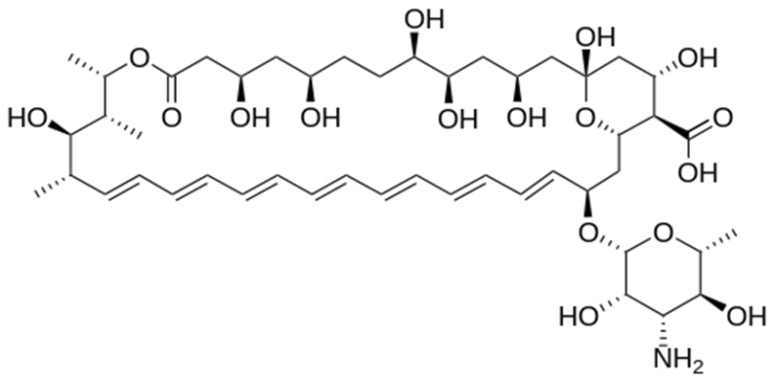
ring instead of an imidazole ring. Like other imidazole- and triazole-class antifungals, Fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase. Mammalian demethylase activity is much less sensitive to fluconazole than fungal demethylase. This inhibition prevents the conversion of lanosterol to ergosterol, an essential component of the fungal cytoplasmic membrane, and subsequent accumulation of 14α-methyl sterols. Fluconazole is primarily fungistatic; however, it may be fungicidal against certain organisms in a dose-dependent manner, specifically *Cryptococcus.*[64]



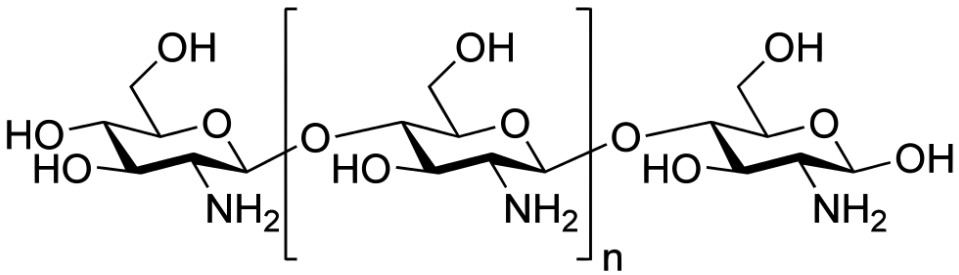
Fluconazole is a synthetic, bistriazole antifungal agent, effective in treating superficial and systemic infections caused by candida species. Fluconazole works by interfering with synthesis of fungal cell membrane. Fluconazole is commonly used to prevent yeast infections in patients undergoing bone marrow transplantation. 0.2% Fluconazole in previous studies showed antifungal efficacy against *C.albicans* and the results were more or less similar to other antifungal agents used.[64]

Amphotericin B is a polyene antifungal agent with activity against wide variety of fungal pathogens. Amphotericin B exerts its antifungal effect by disruption of fungal cell wall synthesis. Amphotericin B is generally considered cidal against

susceptible fungi at clinical relevant concentrations. As with other polyene antifungals, Amphotericin B binds with ergosterol, a component of fungal cell membranes, forming a transmembrane channel that leads to monovalent ion (K^+, Na^+, H^+ and Cl^-) leakage, which is the primary effect leading to fungal cell death.0.2% Amphotericin B in previous studies showed antifungal efficacy against *C.albicans* and but the efficacy is less when compared to 0.2% Fluconazole.[64]



Chitosan, a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is made by treating shrimp and other crustacean shells with the alkali sodium hydroxide.[12]



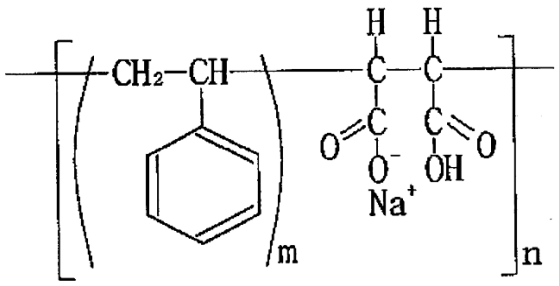
Chitosan is a derivative of chitin, which is commonly found in shells and exoskeletons of some crustacean and is the second most abundant bio-polymer with unique structural and physiologic characteristics.[12] Chitosan exhibits a broad spectrum

of antimicrobial activity by binding to the negatively charged bacterial cellwall followed by attachment to the DNA, inhibiting its replication. Chitosan is a biopolymer having antibacterial properties. Chitosan has high biocompatibility and coagulative properties. It is not an allergen and the antimicrobial activity of this material makes it a suitable choice for use in areas susceptible to infection. The bactericidal effect of chitosan is highly dependent on several factors including its molecular weight and deacetylation degree, physical state, ionic strength, pH, surface characteristics of target microrganisms.[13]

Silver nanoparticles are nanoparticles of silver of between 1 nm and 100 nm in size. Silver nanoparticles have high therapeutic potential. Silver nanoparticles have a wide range of antimicrobial activities and exhibit high performance even at a very low concentration.[14] Introduction of silver into bacterial cells induces a high degree of structural and morphological changes, which can lead to cell death. As the silver nanoparticles come in contact with the bacteria, they adhere to the cell wall and cell membrane. Once bound, some of the silver passes through to the inside, and interacts with phosphate-containing compounds like DNA and RNA, while another portion adheres to the sulphur-containing proteins on the membrane. The silver-sulphur interactions at the membrane cause the cell wall to undergo structural changes, like the formation of pits and pores. Through these pores, cellular components are released into the extracellular fluid, simply due to the osmotic difference. Within the cell, the integration of silver creates a low molecular weight region where the DNA then condenses. Having DNA in a condensed state inhibits the cell's replication proteins contact with the DNA. Thus the introduction of silver nanoparticles inhibits replication and is sufficient to cause the death of the cell. Further increasing their

effect, when silver comes in contact with fluids; it tends to ionize which increases the nanoparticles bactericidal activity. This has been correlated to the suppression of enzymes and inhibited expression of proteins that relate to the cell's ability to produce ATP.[12]

[Chemical Formula 1]



Chitosan- silver nanocomposite is seen to possess a capability of being used as a biosensor as well as in the treatment of cancer as the chitosan present in the nanocomposite is very specific to the cancer cells. Chitosan stabilizes and prolongs the action of silver.[15]

The chitosan suspension was prepared by solubilizing chitosan (1.0 g) in acetic acid (50 mL, 1.0 wt %) solution. Then, $AgNO_3$ (50 mL, 0.01 M) was added immediately into the suspension under constant stirring for 2.0 hours for preparation of the $AgNO_3$ in chitosan suspension. $NaBH_4$ (20 mL, 0.04 M) was added to the suspension of $AgNO_3$/Cts and an immediate color change from pale yellow to brown indicating the formation of AgNPs (20 wt% Ag).[104]

When $AgNO_3$ was mixed with chitosan solution, Ag^+ ions probably bound to chitosan macromolecules via electrostatic interaction between the electron- rich oxygen atoms of the polar hydroxyl and ether groups of chitosan and the

electropositive transition cations (Ag^{+}). Attachment of silver to the nitrogen atoms in chitosan reduced the vibration intensity of the N-H bond due to increased molecular weight after silver binding.[104]

Persistent endodontic infections are mainly due to retention and recolonization of microorganisms in the dentinal tubule. *C.albicans* was selected as the test organism due to the presence of the organism in 7-18% cases of persistent apical periodontitis.[105] It is a dimorphic fungus which has the ability to grow on the dentinal surfaces in the absence of oral tissue fluids and penetrate into the dentinal tubules by its various growth patterns and it is considered as "dentinophilic" microorganism.[4]It has been used extensively in endodontic research because it has been detected in 30% to 45% of post treatment diseases and due to high level of resistance to a wide range of antimicrobial agents. *C.albicans* has been used as the test organism to determine the efficacy of endodontic irrigants in the previous *in vitro* studies. ATCC 10231 strains of *C.albicans* which has been the standard strain used in the previous studies was selected for present study.[106]

In this *in vitro* study, fifty single rooted mandibular premolars freshly extracted for orthodontic purposes were chosen. Teeth with cracks, caries, restorations, resorptive defects and open apices are avoided. The teeth were immersed in 3%NaOCl (freshly prepared) for 15 minutes to remove debris and organic tissue. Then teeth were cleaned using an ultrasonic scaler to render them free from calculus and tissue tags. Finally teeth were stored in 0.2% sodium azide until use. The teeth were then decoronated at the cementoenamel junction using diamond disc. This was done to standardize the root length. A 25mm size 15 stainless steel K-file was inserted into the root canal until it was seen at the apical foramen. Working length was

obtained by subtracting one millimetre from this length. Gates Glidden drills 1 to 4 was used for coronal flaring and apical preparation was done until ISO size 50. The roots were coated with two coats of nail varnish and apical foramen sealed with Type II GIC to prevent extrusion of irrigants. Subsequently, the roots were sterilized in an autoclave for 15min, at 121°C and 15 lb pressure.

A suspension of *Candida albicans* was adjusted to 0.5% turbidity on the McFarland scale (1.5×10^{8}bacteria/mL).The canals of the experimental teeth was cautiously inoculated with 0.5mL of the freshly prepared suspension and was stored in a glass test tube. The samples were stored and incubated at 37°C and 91% humidity for 96 hours. Every 24 hours, the vials containing the experimental teeth were replenished with freshly prepared suspension of *Candida albicans*. After 48 hours, aliquots was taken from each tooth using a syringe and plated on 4% Sabouraud dextrose agar plate to verify the growth of *Candida albicans*. At the end of 96 hours, teeth were removed from the glass test-tube vials. Finally excess fluid in the canal was removed with sterile paper points.

The protocol followed for irrigation was as follows: Group I (control group) in which irrigation was done with 5.25%NaOCl, 17%EDTA and 0.9% Saline. Group II was irrigated with 5.25%NaOCl, 17%EDTA, Chitosan-Silver Nanocomposite (20%Ag) and 0.9% Saline. Group III was irrigated with 5.25%NaOCl, 17%EDTA, 0.2%Fluconazole and 0.9% saline. Group IV was irrigated with 5.25% NaOCl, 17% EDTA, 1% Clotrimazole and 0.9% Saline. Group V was irrigated with 5.25%NaOCl, 17%EDTA, 0.2% Amphotericin B and 0.9% Saline.

The time of contact of each irrigant was 1 minute and final flush with 5mL of distilled water. The antifungal agents were injected into the root canal by using a 26 gauge needle. The time of contact was 5 minutes; after which, the sample was flushed with 5mL of distilled water to prevent carryover of irrigants. In most of the *in vitro* studies, sample preparation was done using burs of different sizes, Gates glidden drills, K files, paper points. In this study sample preparation was done using 50 size K files which was similar to the sample collection done in a previous study in 2010.[51]The methodology adopted for enumeration of CFU was in resemblance with the study conducted by Lynn *et al*.[98]

In this present study a modification of Haapasalo and Orstavik model was used for assessing the antimicrobial efficacy of endodontic irrigants in dentinal tubule disinfection.[107]The model was further modified by using extracted human teeth rather than bovine teeth. This modification was appropriate because of the marked difference in diameter between the canals of bovine and human teeth.[103] Quantitative estimation of CFU in the dentinal tubules after disinfection was included in the present study which was a modification of Haapasalo and Orstavik model.[108]

In the present study all experimental teeth were flushed with 15mL of sterile saline and canals were dried with sterile absorbent paper points. A small amount of saline solution was introduced into the canal, and then an endodontic hand file was used in a filing motion to a level approximately 1mm short of the root apex. A 1µm inoculation loop was used to remove aliquots from the fluid and was plated on Sabouraud 4% dextrose agar. The plates were incubated at 36°C and 91% humidity for 48 hours. After the incubation period, the growth of *Candida albicans* was

assessed with light microscopy at 400X. The number of Colony Forming Units (CFUs) of candida serves as a measure of the antifungal activity.

Data entry was done in Microsoft excel. The values obtained were tabulated and statistically analyzed using computer software SPSS (16.0) version. The data was expressed in its mean and standard deviation. One way ANOVA was applied for statistical analysis. Post hoc followed by Dunnet 't' test was used to find the statistical significance between the groups. P value less than 0.05 ($P<0.05$) was considered statistically significant at 95% confidence interval.

In this study, four antifungal agents Chitosan- silver nanocomposite, 1%Clotrimazole, 0.2% Fluconazole, 0.2% Amphotericin B were used to irrigate the tooth specimens in Groups II,III,IV and V respectively and their antifungal efficacy against *Candida albicans* was assessed. The CFU was counted through light microscopy and the results were compared with the control group. Group II (Chitosan-silver nanocomposite) and Group IV (1% Clotrimazole) showed better antifungal efficacy when compared to other antifungal agents and shows equal efficacy that of Control Group. Complete inhibition of growth of *Candida albicans* was shown by Group I, Group II and Group IV. Colony counting units (CFU) was shown by Group III (0.2% Fluconazole) and Group V (0.2% Amphotericin B) proved their fewer efficacies in inhibition of growth of *candida*.

1% Clotrimazole (Group IV) irrigated specimens showed complete inhibition of growth of *C.albicans* and showed similar antifungal effect with Group I and Group II. Smith *et al.* evaluated the antifungal activity of Clotrimazole (Bay b 5097) compared with those of Amphotericin B, Griseofulvin, Nystatin, and Pyrrolnitrin.

They reported that Clotrimazole is less active than Amphotericin B against *Candida albicans* and *Aspergillus fumigatus*. The activity of Clotrimazole against dermatophytes is comparable to that of pyrrolnitrin and superior to that of either Nystatin or Griseofulvin. More importantly, the ranges of inhibitory and fungicidal concentrations are narrow with little deviation either within or between species.[17] Wadhwani *et al*. evaluated the antifungal efficacy of 5.25% Sodium Hypochlorite, 2% Chlorhexidine gluconate, and 17% EDTA as final irrigant with and without the inclusion of an Antifungal agent that is 1% Clotrimazole on *Candida albicans*. They concluded that 5.25% Sodium hypochlorite exhibited superior antifungal efficacy compared to 2% Chlorhexidine gluconate and 17% EDTA. On inclusion of 1% Clotrimazole, there was a significant decrease in colony forming units. 5.25% Sodium hypochlorite and 2% Chlorhexidine gluconate with Clotrimazole showed significantly greater antifungal properties than 17% EDTA with Clotrimazole.[73]

0.2% Fluconazole (Group III) and 0.2% Amphotericin B (Group V) irrigated specimens did not show significant difference in inhibition of growth of *C.albicans* and this two antifungal agents showed less efficacy compared to Group I,II and IV. But when comparing Group III with group V, 0.2% Fluconazole showed more antifungal activity than 0.2% Amphotericin B**.** Ignatius *et al.* evaluated the efficiency of sodium hypochlorite and four other intra canal medicaments in eliminating the *Candida albicans* in the root canal system. They concluded that irrigation with 2.5% NaOCl was effective in 90% of the samples, followed in decreasing order of effectiveness by Amphotericin B powder and distilled water (80% effectiveness), ZnO powder and 2% CHX (70% effectiveness), $Ca(OH)_2$ powder and 2% CHX (60% effectiveness), $Ca(OH)_2$ powder and saline (50% effectiveness), and saline + no

intracanal medication.[71] Dumani *et al.* evaluated antimicrobial activity of 4 antibiotic agents (for *E.faecalis*) and 4 antifungal agents (for *C.albicans*) by agar dilution method. Organisms were collected from root canals. They concluded that for *C.albicans,* 18 isolates were susceptible to amphotericin B, nystatin, fluconazole but showed resistance to ketoconazole.[109]

Chitosan-silver nanocomposite (Group II) showed complete inhibition of growth of *Candida albicans* and did not show significant difference with Group I (control) and Group IV (1% clotrimazole). Kanikireddy *et al.* evaluated the anti-microbial and anti-fungal activity of the chitosan- PVA silver nanoparticle films and had demonstrated significant effects against *E. coli, Pseudomonas, Staphylococcus, Micrococcus, Candida albicans,* and *P. aeruginosa.* To improve further their therapeutic efficacy as anti-microbial agents, curcumin encapsulated chitosan-PVA silver nanocomposite films are developed which showed enormous growth inhibition of *E. coli* compared to curcumin and chitosan-PVA silver nanoparticles film alone.[63] Solmaz *et al.* evaluated the antibacterial properties and characterization of chitosan-silver nanocomposite materials. They concluded that Antibacterial effect of chitosan-silver nanoparticle materials was increased by increasing Ag amount of the composite materials. The presence of small amount of metal nanoparticles in the composite was enough to significantly enhance antibacterial activity as compared with pure chitosan.[83]

An important limitation of many studies when evaluating the endodontic microbiota refers to sample preparation. In comparison to the study conducted by Berber *et al.* 5.25% NaOCl eliminated ATCC 29212 strains of *E. faecalis* in a 10 minute contact time.[110]The methodology adopted in the latter study is similar to the

present study except for the sample preparation. Burs of different sizes were used to procure dentinal shavings unlike K-files used in the present study. Bacterial inhibition at greater depths was assessed using burs of different sizes whereas K files were indicative of bacterial inhibition to a limited depth. Increasing the concentration is undesirable because it is an irritant to periapical tissue.[111]Other undesirable effects of NaOCl are its unpleasant taste, high toxicity, corrosive to instruments, reduces the elastic modulus and flexural strength of dentin.[112]Test irrigants used in this study was adequate to classify nanoformulations as antimicrobial agents against *C.albicans*. The active compounds imparting the antimicrobial effect of chitosan-silver nanocomposite would have been assessed if a FTIR was performed. Analysis of the presence of functional groups which are responsible for the antifungal efficacy of chitosan silver nanocomposite can be better determined by FTIR. Chitosan silver nanocomposite should contain 20 wt% silver nanoparticles for better antifungal property and the percentage of silver nanoparticles can be assessed with FTIR analysis.[104]To recommend nanoformulations as endodontic irrigants, it has to satisfy all the ideal properties of an irrigant. But in the present study only the antimicrobial property has been studied. With a deeper knowledge of other properties of nanoformulations and if found to satisfy the properties of an ideal irrigant it could open doors to a new era in endodontics.

SUMMARY & CONCLUSION

Complete debridement and disinfection of the pulpal space is considered to be essential for predictable long term success in endodontic treatment. Eliminating microorganisms from root canal system is possible only by a thorough Chemomechanical preparation. However complete sterilization of pulp space is not always achieved due to extremely complex anatomy. *C.albicans* is the most common species isolated in root filled teeth with apical periodontitis. The constant increase in antibiotic resistant strains and side effects caused by synthetic drugs has prompted researchers to look for nanoformulations. The purpose of this study was to evaluate the antifungal efficacy of chitosan-silver nanocomposite as endodontic irrigant against *C.albicans*. Extracted human teeth were biomechanically prepared, autoclaved and inoculated with *C.albicans* and incubated for 96 hours. After 96 hours the teeth were randomly divided into five groups and treated with respective irrigants for 5 minutes. Aliquots collected using inoculation loops. Antimicrobial efficacy was indicated by reduction in CFU. 5.25% NaOCl, 1% Clotrimazole and Chitosan silver nanocomposite showed complete inhibition in all samples tested. 0.2% Amphotericin B and 0.2% Fluconazole showed reduction in CFU in all samples tested and there was complete inhibition in 8 samples of Amphotericin B and 9 samples of Fluconazole. The results are indicative of potent antimicrobial action. The use of chitosan- silver nanocomposite as a root canal irrigant might prove to be advantageous considering the several undesirable characteristics of standard irrigants and other frequently used antimicrobials. Further research is warranted to conclusively recommend nanoformulations as a root canal irrigant.

Standard irrigants 5.25% NaOCl and 17% EDTA showed complete inhibition and remain as standard irrigants. With ever increasing resistance to synthetic drugs and typical features of *C.albicans,* nanocomposite can be an alternative option provided all the ideal properties of an irrigant are satisfied.

TABLES

Table-1: Mean antifungal activity of different groups

Groups	Type of irrigation	Mean (CFU/µl) (MEAN±SD)
Group-I	control	0.00±0.00
Group-II	Chitosan-silver nanocomposite	0.00±0.00
Group-III	0.2% Fluconazole	0.30±0.48
Group-IV	1% Clotrimazole	0.00±0.00
Group-V	0.2% Amphotericin B	0.40±0.96

Table-2: Comparison of antifungal activity of Group-I with other groups

Groups	Mean (CFU/µl) (MEAN±SD)	P value
Group-I	0.00±0.00	
Group-II	0.00±0.00	1.00
Group-III	0.30±0.48*	0.02
Group-IV	0.00±0.00	1.00
Group-V	0.40±0.96*	0.01

(*P<0.05 significant when compared Group-I with other groups)

Table-3: Comparison of antifungal activity of Group-II with other groups

Groups	Mean (CFU/µl) (MEAN±SD)	P value
Group-II	**0.00±0.00**	
Group-I	**0.00±0.00**	**1.00**
Group-III	**0.30±0.48***	**0.02**
Group-IV	**0.00±0.00**	**1.00**
Group-V	**0.40±0.96***	**0.01**

(*P<0.05 significant when compared Group-II with other groups)

Table-4: Comparison of antifungal activity of Group-III with other groups

Groups	Mean (CFU/µl) (MEAN±SD)	P value
Group-III	**0.30±0.48**	
Group-I	**0.00±0.00***	**0.02**
Group-II	**0.00±0.00***	**0.02**
Group-IV	**0.00±0.00***	**0.02**
Group-V	**0.40±0.96***	**0.03**

(*P<0.05 significant when compared Group-III with other groups)

Table-5: Comparison of antifungal activity of Group-IV with other groups

Groups	Mean (CFU/µl) (MEAN±SD)	P value
Group-IV	0.00±0.00	
Group-I	0.00±0.00	**1.00**
Group-II	0.00±0.00	**1.00**
Group-III	0.30±0.48*	**0.02**
Group-V	0.40±0.96*	**0.01**

(*P<0.05 significant when compared Group-IV with other groups)

Table-6: Comparison of antifungal activity of Group-V with other groups

Groups	Mean (CFU/µl) (MEAN±SD)	P value
Group-V	**0.40±0.96***	
Group-I	**0.00±0.00***	**0.01**
Group-II	**0.00±0.00***	**0.01**
Group-III	**0.30±0.48***	**0.03**
Group-IV	**0.00±0.00***	**0.01**

(*P<0.05 significant compared Group-V with other groups)

Table-7: Multiple comparison of antifungal activity of different groups

Groups	Type of irrigation	Mean (CFU/µl) (MEAN±SD)
Group-I	**CONTROL**	**0.00±0.00**
Group-II	**Chitosan-silver nanocomposite**	**0.00±0.00**
Group-III	**0.2% Fluconazole**	**0.30±0.48*,#**
Group-IV	**1% Clotrimazole**	**0.00±0.00$**
Group-V	**0.2% Amphotericin B**	**0.40±0.96*,#,$,ǁ**

(*P<0.05 significant compared Group-I with other groups, #P<0.05 significant compared Group-II with other groups, $P<0.05 significant compared Group-III with other groups, ǁP<0.05 significant compared Group-IV with other groups)

FIGURES

Fig. 1. ARMAMENTARIUM

Fig .2. MAGNETIC STIRRER

Fig. 3. LAMINAR AIR FLOW

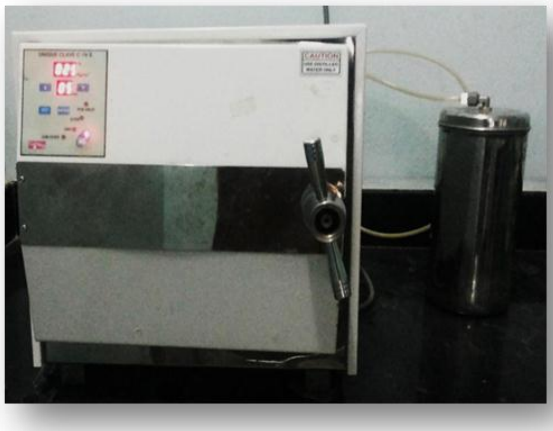


Fig .4. AUTOCLAVE

Fig. 5. INCUBATOR

Fig. 6(a). FIFTY FRESHLY EXTRACTED PREMOLAR TEETH

Fig. 6(b).DECORONATION

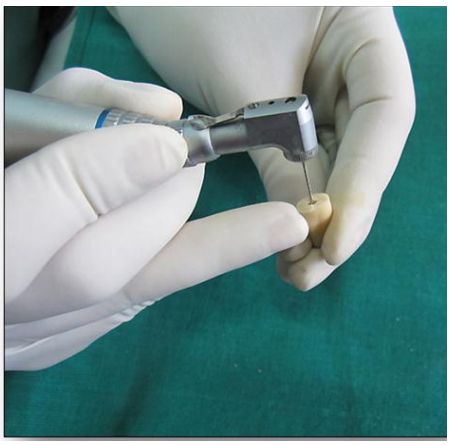


Fig. 6(c). CORONAL FLARING WITH GATES GLIDDEN DRILL

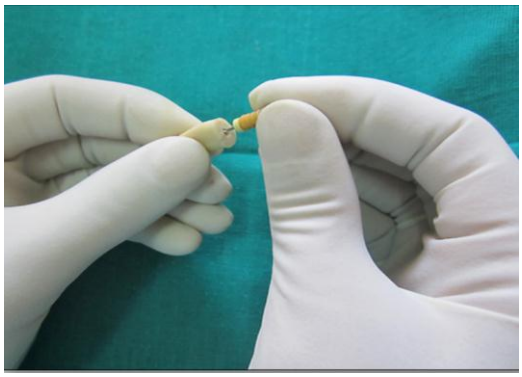


Fig.6(d). INSTRUMENTATION

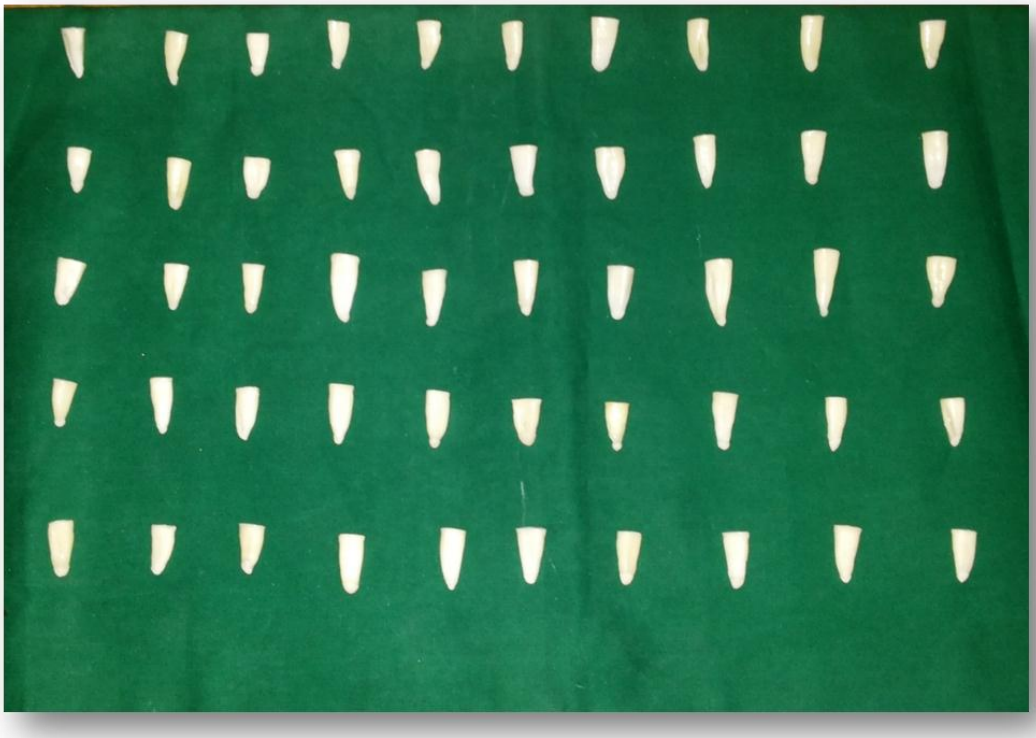


Fig 7. FIFTY DECORONATED SPECIMENS

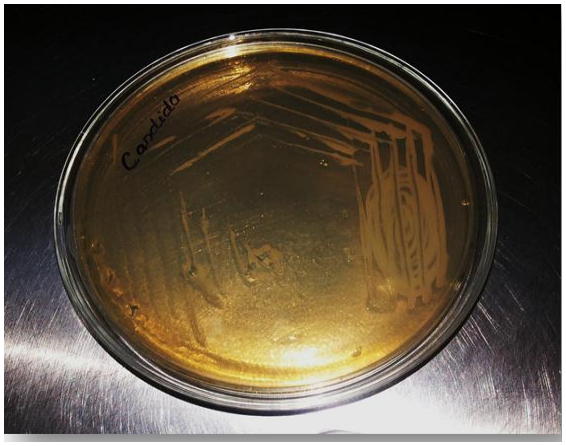


Fig. 8(a). *CANDIDA ALBICANS* ATCC 10231 STRAIN

Fig. 8(b). 0.5% McFARLAND TURBIDITY

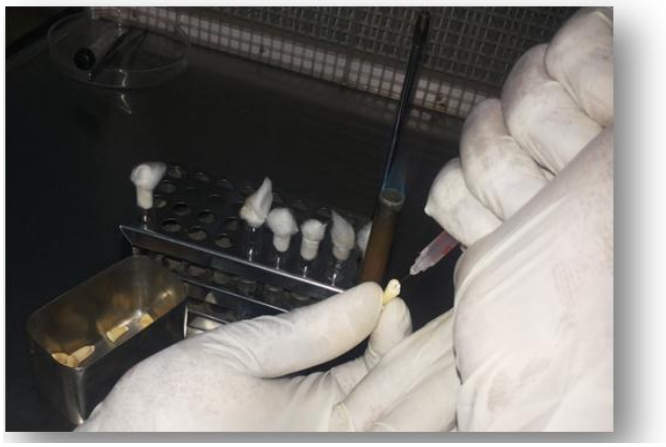


Fig .8(c). *CANDIDA ALBICANS* INOCULATION INTO THE TOOTH

Fig. 9. SAMPLES IN INCUBATOR

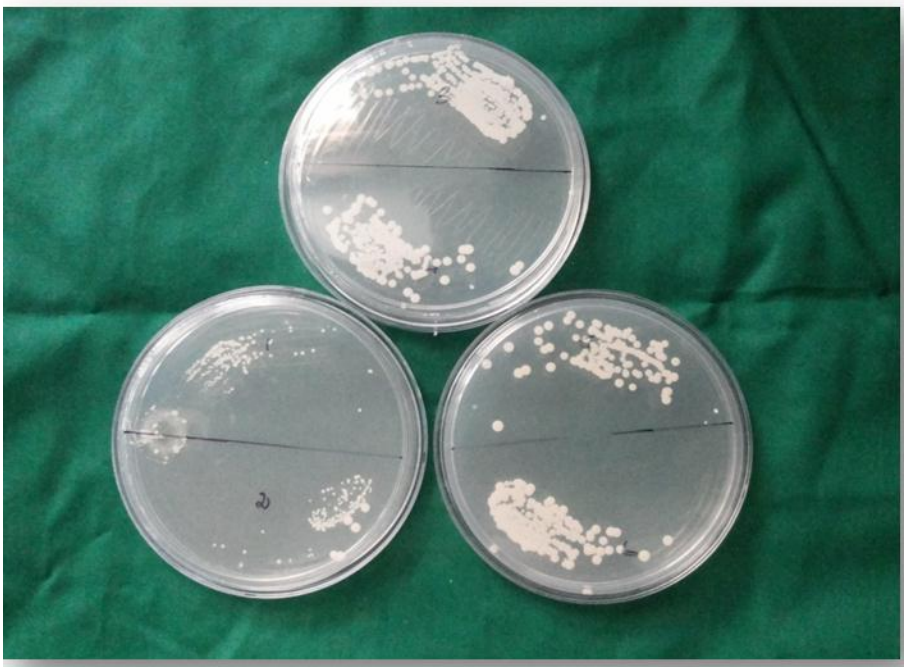


Fig.10. VERIFICATION OF GROWTH OF *CANDIDA ALBICANS* AFTER 48 HOURS

GROUPS

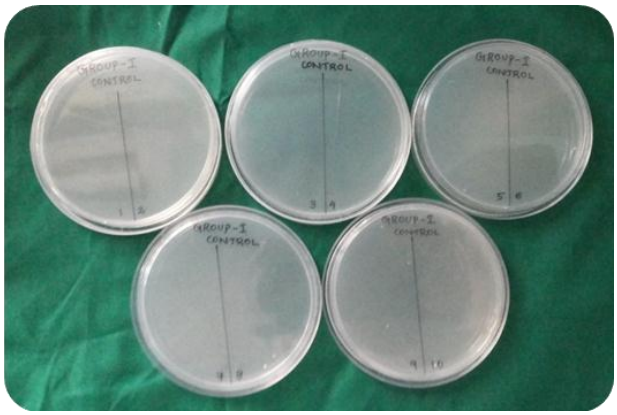


Fig.11(a).GROUP -I

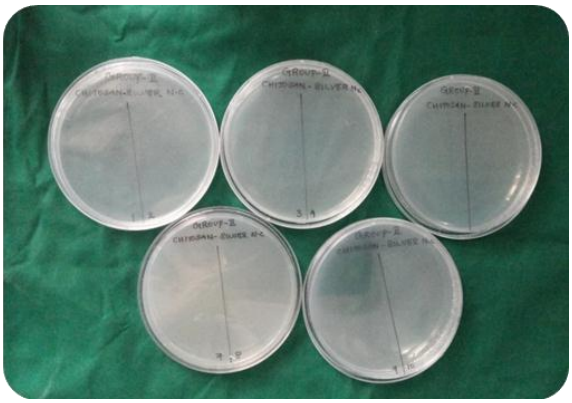


Fig.11(b).GROUP -II

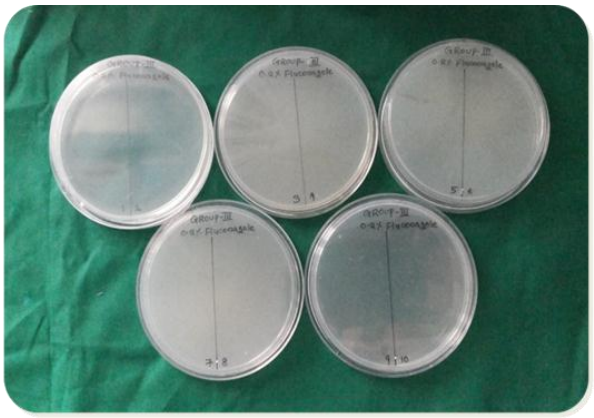


Fig.11(c). GROUP- III

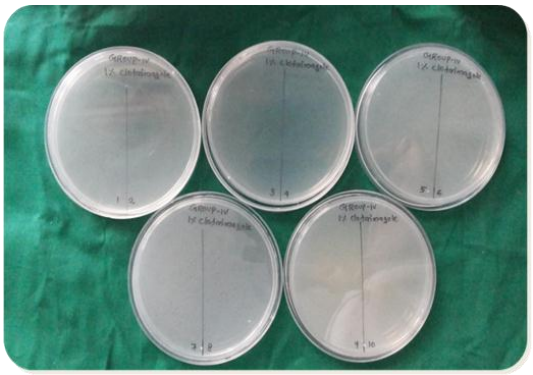


Fig.11(d). GROUP- IV

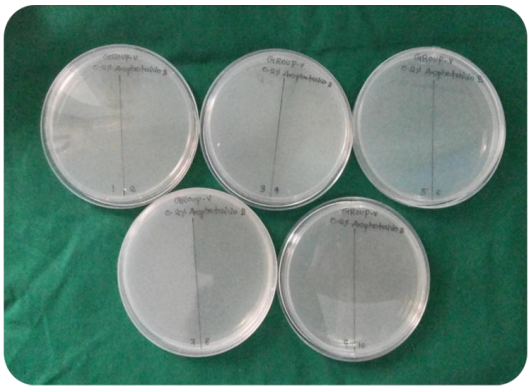


Fig.11(e). GROUP- V

Fig.12.ANTIFUNGAL AGENTS

Fig.13. IRRIGATION OF THE TOOTH

AFTER INCUBATION

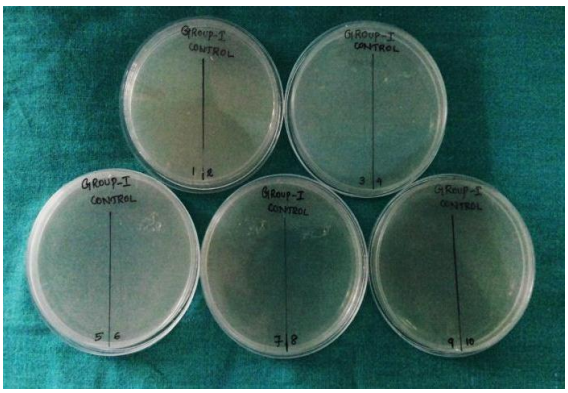


Fig.14(a).GROUP -I

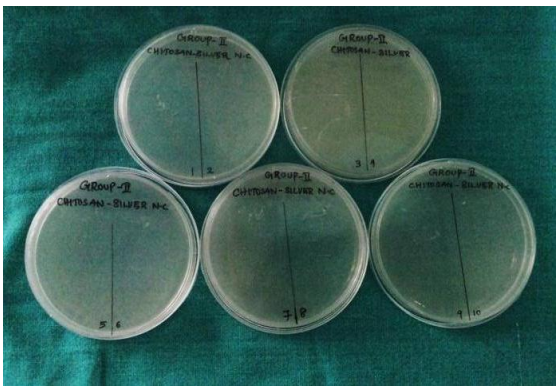


Fig.14(b).GROUP -II

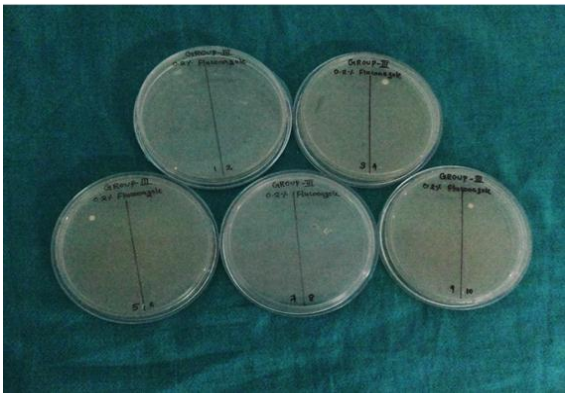


Fig.14(c). GROUP -III

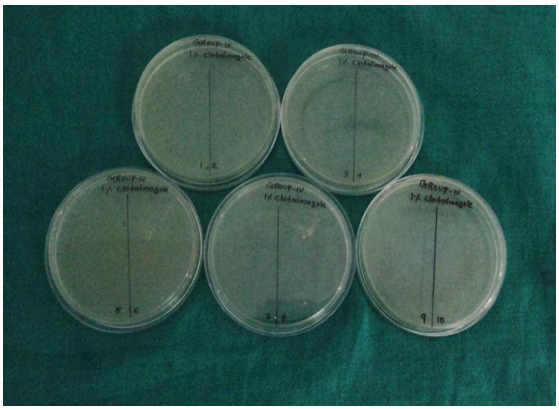


Fig.14(d).GROUP -IV

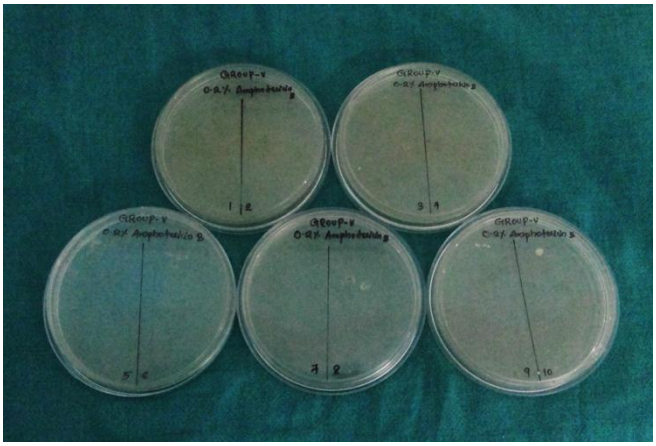


Fig.14(e).GROUP-V

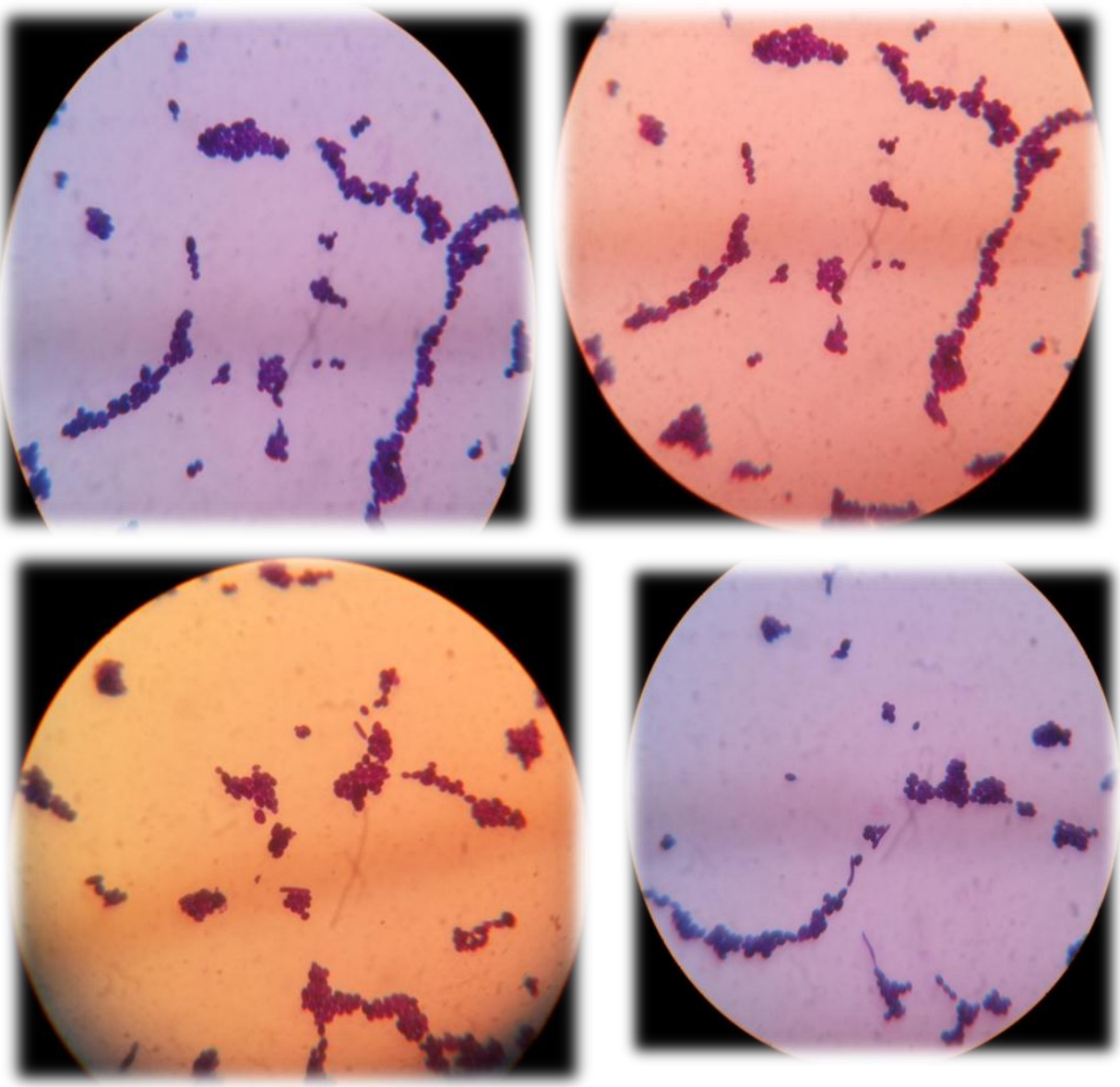


Fig.15.LIGHT MICROSCOPIC VIEW 4OOX (0.2% FLUCONAZOLE)

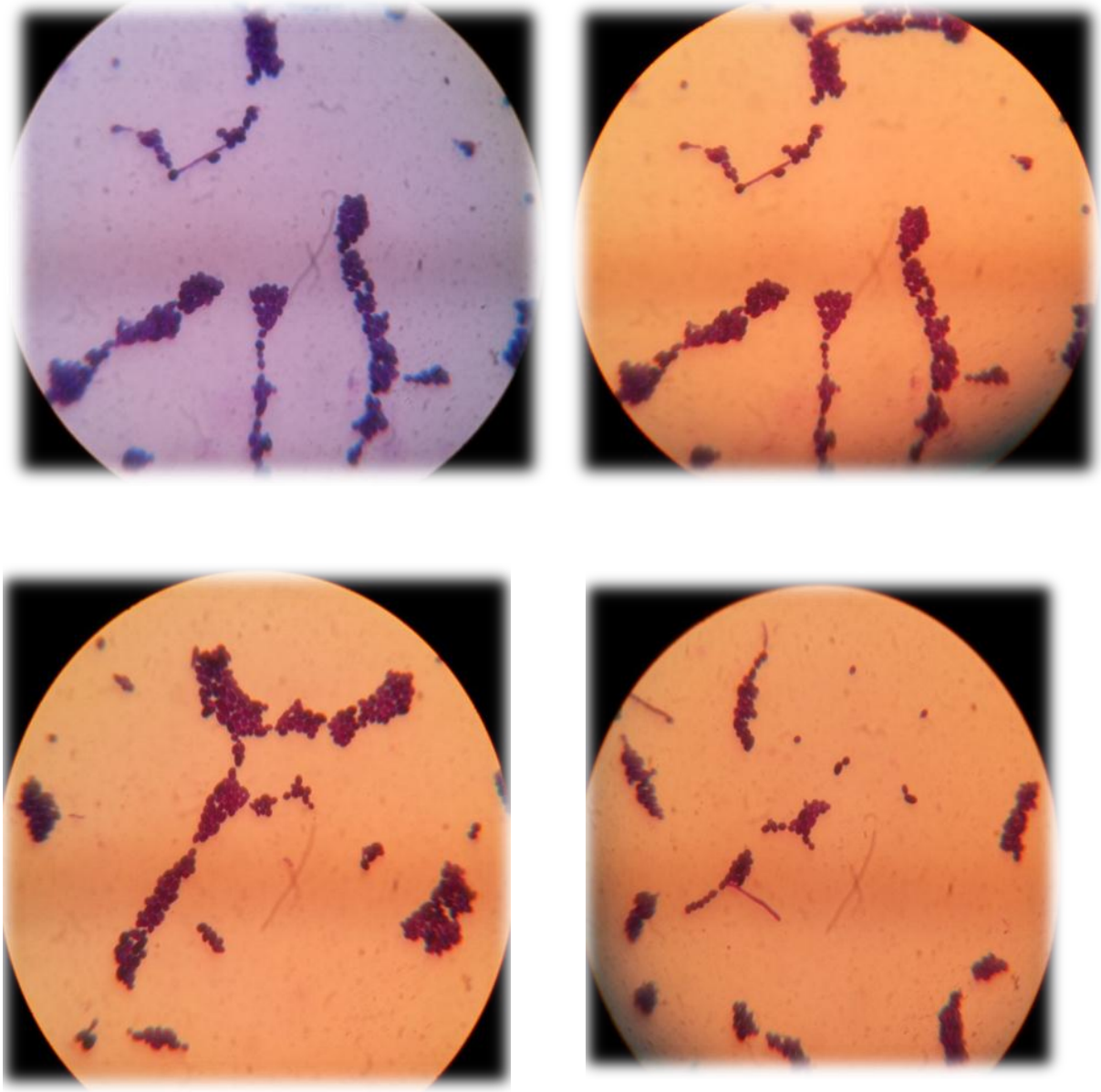


Fig.16.LIGHT MICROSCOPIC VIEW 4OOX (0.2% AMPHOTERICIN B)

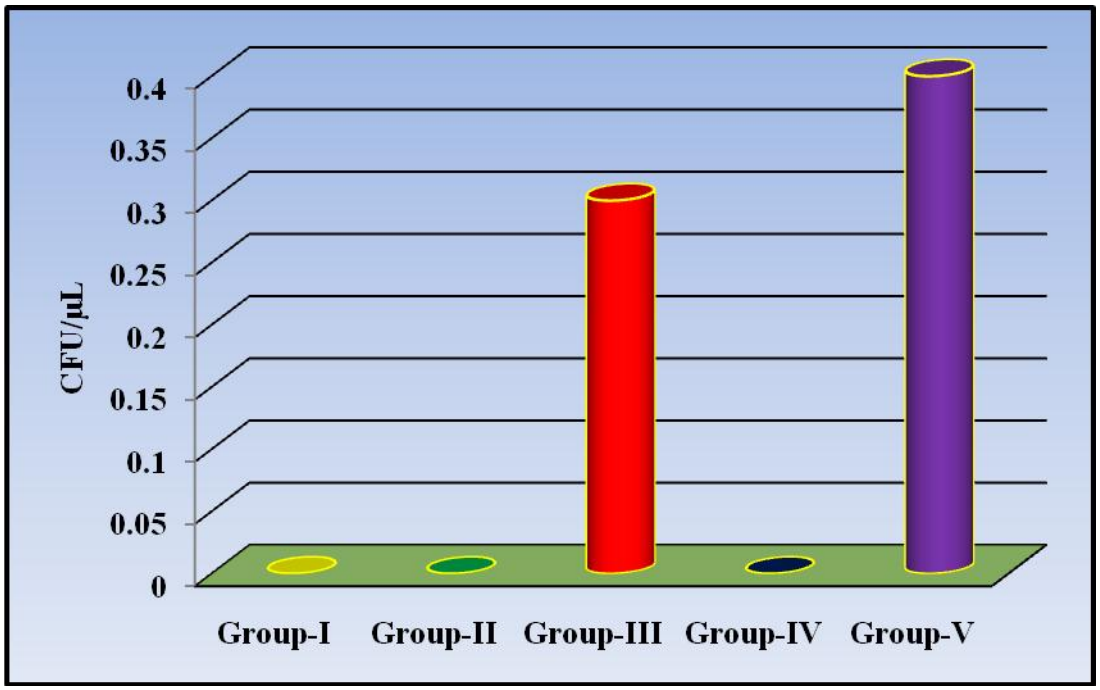


Fig.17 : Graphical representation of multiple comparison of antifungal activity (CFU/µl) of different groups

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